THE SODIUM-HYDROGEN EXCHANGER

From Molecule To Its Role In Disease

edited by
Morris Karmazyn
Metin Avkiran
Larry Fliegel

KLUWER ACADEMIC PUBLISHERS

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Morris Karmazyn
University of Western Ontario
London, Ontario, Canada

Metin Avkiran
King's College London
United Kingdom

Larry Fliegel
University of Alberta
Edmonton, Alberta, Canada

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Table of Contents

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

pH-REGULATORY MECHANISMS IN THE MAMMALIAN OOCYTE AND EARLY EMBRYO

Chapter 9

Na^+/H^+ EXCHANGER ACTIVATION BY MYOCARDIAL STRETCH

Chapter 10

THE PARADOXICAL ROLE OF Na^+/H^+ EXCHANGER IN THE DIABETIC HEART

Chapter 11

ROLE OF Na-H EXCHANGER IN VASCULAR REMODELLING IN DIABETES

Chapter 12

THE POTENTIAL ROLE OF THE Na^+/H^+ EXCHANGER IN ISCHEMIA/REPERFUSION INJURY OF THE CENTRAL NERVOUS SYSTEM

Chapter 13

Chapter 14

Chapter 15

Chapter 16

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 8

Chapter 9

Chapter 10

Chapter 11

Chapter 12

Chapter 14

Chapter 15

Chapter 16

Chapter 17

Chapter 18

Chapter 20

LIST OF TABLES

Chapter 2

Chapter 5

List of Contributors

(email address provided for corresponding authors)

Metin Avkiran
Centre for Cardiovascular Biology and Medicine
King's College London
The Rayne Institute
St Thomas' Hospital
London SE1 7EH
United Kingdom
metin.avkiran@kcl.ac.uk

Iyad M Ayoub
Department of Medicine
Finch University of Health Sciences
The Chicago Medical School
North Chicago VA Medical Center
3001 Green Bay Road
North Chicago, Illinois 60064
USA

Jay M Baltz
Ottawa Health Research Institute
Loeb Building
725 Parkdale Ave
Ottawa, Ontario K1Y 4E9
Canada
jbaltz@ohri.ca

Norbert Beier
Diab & Compl Res DA
Merck KGaA
Frankfurter Str 250
D-64271 Darmstadt
Germany

Henry J Binder
Departments of Internal Medicine
Cellular and Molecular Physiology and Surgery
Yale University
New Haven, Connecticut 06520
USA
henry.binder@yale.edu

María C Camilión de Hurtado
Centro de Investigaciones Cardiovasculares
Facultad de Ciencias Médicas
60 y 120 (1900) La Plata
Argentina

Horacio E Cingolani
Centro de Investigaciones Cardiovasculares
Facultad de Ciencias Médicas
60 y 120 (1900) La Plata
Argentina
cicmes@infovia.com.ar

Rodney J Dilley
Morphology Laboratory
Baker Medical Research Institute
Melbourne, Vic 8008
Australia

Irene L. Ennis
Centro de Investigaciones Cardiovasculares
Facultad de Ciencias Médicas
60 y 120 (1900) La Plata
Argentina

Danielle Feuvray
Physiologie Cellulaire
UMR CNRS 8078,
Université Paris-Sud XI
Bât 443
91405 Orsay Cedex
France
danielle.feuvray@ibaic.u-psud.fr

Larry Fliegel
Department of Biochemistry
University of Alberta
Edmonton, Alberta T6G 2H7
Canada
lfliegel@ualberta.ca

Livnat Galili
Division of Microbial and
Molecular Ecology
Institute of Life Sciences
The Hebrew University of
Jerusalem
91904 Jerusalem
Israel

Raúl J Gazmuri
Department of Medicine
Finch University of Health
Sciences
The Chicago Medical School
North Chicago VA Medical
Center
3001 Green Bay Road
North Chicago, Illinois 60064
USA
Raul.Gazmuri@med.va.gov

John Geibel
Departments of Internal
Medicine
Cellular and Molecular
Physiology and Surgery
Yale University
New Haven, Connecticut 06520
USA

Sergio Grinstein
Programme in Cell Biology
The Hospital for Sick Children
Research Institute
Toronto, Ontario M5G 1X8
Canada

Garrett J Gross
Department of Pharmacology
and Toxicology
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, Wisconsin 53226
USA
ggross@mcw.edu

Robert S Haworth
Centre for Cardiovascular
Biology and Medicine
King's College London
The Rayne Institute
St Thomas' Hospital
London SE1 7EH
United Kingdom

Katia Herz
Division of Microbial and
Molecular Ecology
Institute of Life Sciences
The Hebrew University of
Jerusalem
91904 Jerusalem
Israel

Takashi Hisamitsu
Department of Molecular
Physiology
National Cardiovascular
Research Institute
Fujishirodai 5-7-1, Suita
Osaka 565-8565
Japan

Morris Karmazyn
Department of Physiology and
Pharmacology
University of Western Ontario
Medical Sciences Building
London, ON N6A 5C1
Canada
Morris.Karmazyn@fmd.uwo.ca

Hans Jochen Lang
Aventis Pharma Deutschland
GmbH
Building G878
65926 Frankfurt am Main
Germany
HansJochen.Lang@aventis.com

Peter J Little
Cell Biology of Diabetes
Laboratory
Baker Medical Research Institute
St. Kilda Road Central
PO Box 6492
Melbourne, Vic 8008
Australia
Peter.Little@baker.edu.au

Marcus Müller
Division of Microbial and
Molecular Ecology
Institute of Life Sciences
The Hebrew University of
Jerusalem
91904 Jerusalem
Israel

M Lee Myers
Division of Cardiovascular
Surgery
London Health Sciences
Centre-Victoria
University of Western Ontario
London, Ontario N6A 4G5
Canada
Ml.Myers@lhsc.on.ca

John Orlowski
Department of Physiology
McGill University
Montreal, Quebec H3G 1Y6
Canada
john.orlowski@mcgill.ca

Michael H O'Regan
Biomedical Sciences
School of Dentistry
University of Detroit Mercy
8200 W. Outer Drive
Detroit, Michigan 48219
USA

Etana Padan
Division of Microbial and
Molecular Ecology
Institute of Life Sciences
The Hebrew University of
Jerusalem
91904 Jerusalem
Israel
etana@vms.huji.ac.il

Tianxiang Pang
Department of Molecular
Physiology
National Cardiovascular
Research Institute
Fujishirodai 5-7-1, Suita
Osaka 565-8565
Japan

Néstor G. Pérez
Centro de Investigaciones
Cardiovasculares
Facultad de Ciencias Médicas
60 y 120 (1900) La Plata
Argentina

John W Phillis
Department of Physiology
School of Medicine
Wayne State University
540 E. Canfield Ave
Detroit, Michigan 48201
USA
jphillis@med.wayne.edu

Julie G Pilitsis
Department of Neurological
Surgery
School of Medicine
Wayne State University
4201 St. Antoine
Detroit, Michigan 48201
USA

Vazhaikkurichi M Rajendran
Departments of Internal
Medicine
Cellular and Molecular
Physiology and Surgery
Yale University
New Haven, Connecticut 06520
USA

Abraham Rimon
Division of Microbial and
Molecular Ecology
Institute of Life Sciences
The Hebrew University of
Jerusalem
91904 Jerusalem
Israel

Wolfgang Scholz
Diab & Compl Res DA
Merck KGaA
Frankfurter Str 250
D-64271 Darmstadt
Germany
Wolfgang.Scholz@merck.de

Munekazu Shigekawa
Department of Molecular
Physiology
National Cardiovascular
Research Institute
Fujishirodai 5-7-1, Suita
Osaka 565-8565
Japan

Kenneth W Spitzer
Nora Eccles Harrison
Cardiovascular Research and
Training Institute
University of Utah
Salt Lake City, Utah 84112
USA
spitzer@cvrti.utah.edu

Tzvi Tzubery
Division of Microbial and
Molecular Ecology
Institute of Life Sciences
The Hebrew University of
Jerusalem
91904 Jerusalem
Israel

Richard D Vaughan-Jones
Burdon Sanderson Cardiac
Science Centre
University Laboratory of
Physiology
Parks Road
Oxford, OX13PT
United Kingdom

Shigeo Wakabayashi
Department of Molecular
Physiology
National Cardiovascular
Research Institute
Fujishirodai 5-7-1, Suita
Osaka 565-8565
Japan
wak@ri.ncvc.go.jp

Foreword

I am extremely honored and pleased to have the opportunity to write a few introductory words for this timely volume on Na^+/H^+ exchange. This is a field of investigation that I entered into by challenge and necessity, embraced with passion and finally left in my quest for new discoveries in growth control.

Ten years, one third of my scientific life, has been devoted to uncovering the mysteries of intracellular pH (pH_i) regulation with respect to growth factor action. I got started on this new topic in 1980, when I heard a rather provocative hypothesis presented by Enrique Rozengurt at an ICN-UCLA Keystone meeting on "Cell Surface and Malignancy". He showed that all mitogens induced amiloride-sensitive Na^+ entry into resting cells and proposed that, if a compound stimulates Na^+ influx, it could be a mitogen. In support of his proposal Enrique reported that the amphipathic polypeptide, mellitin, which induced Na^+ influx, was indeed mitogenic for 3T3 cells. This was only correlation at this stage. However, I was fascinated by this talk. I immediately approached Enrique to inform him of my skepticism about this beautiful story, and to indicate that I would only be convinced when I succeeded in isolating mutant fibroblasts lacking the amiloride-sensitive Na^+ transporter. "Good luck!" was his response.

I took Enrique's "good luck" wish to mean that he was not going to compete with us on genetics, which was great! At the same Keystone meeting, I had presented the properties of a glycolysis-deficient fibroblast mutant (phosphoglucose isomerase), demonstrating that both increased aerobic glycolysis and glucose transport were not essential for the transformed phenotype. Mutant fibroblasts impaired in aerobic glycolysis developed tumors in nude mice with the same incidence as wild type cells. This simple genetic approach killed the Warburg hypothesis. I was therefore confident that somatic cell genetics could be an efficient approach in dissecting growth control mechanisms. Although I knew the risk and the price (at least one to two years of work), I was eager and convinced that it was the right time to attack another important issue in growth control. My goal was to evaluate the role of this mitogen-induced Na^+ flux mechanism and the associated pH_i alkalinization in both growth factor action and cell cycle progression.

At that time the "Yale School of Physiology", with the pioneering work of Peter Aronson, Walter Boron and others, illuminated the field of transport and pH_i regulation in higher eukaryotes. Their advances greatly facilitated the progress of Sonia Paris and Gilles L'Allemain, working in my group, in understanding the biochemistry and functionality of the amiloride-sensitive Na^+/H^+ antiporter. This step was a prerequisite for establishing a genetic screen. The reversibility, ion selectivity and

allosteric activation of the antiporter by intracellular H^+ provided the basis for my inspiration for "H^+ suicide selection". With this killing method and specific genetic screen in hand, it was a simple delight to isolate mammalian cells lacking a functional Na^+/H^+ exchanger and to show that pH_i, regulated via the growth factor-activatable NHE1, truly did control cell cycle progression. The next step was to get the sequence and structure of this molecule, and it took the time of Claude Sardet's PhD thesis to complete the relevant work. Functional complementation with human genomic DNA of a mouse cell line lacking the antiporter led to the identification of the first human molecule, NHE1.

This book is entitled "The Sodium-Hydrogen Exchanger: From Molecule to its Role in Disease". I am glad to have, with my group, contributed to the identification of the Molecule and I am particularly pleased to see how this field has expanded beautifully since we left it.

Jacques Pouysségur
Director of the Institute of Signaling
Developmental Biology and Cancer Research
Nice, France

Preface

The concept of a mammalian sodium-hydrogen exchanger was first proposed in 1961 when Peter Mitchell, a British biochemist and Nobel Laureate (Chemistry, 1978), postulated its existence to explain his chemiosmotic hypothesis. Six years later, Mitchell demonstrated the presence of a sodium-hydrogen exchanger in liver mitochondria. In 1976, Heini Murer and colleagues reported the first identification of a sodium-hydrogen exchanger in cell membranes from mammalian intestine and kidney. As we now know, the sodium-hydrogen exchanger, or NHE (Na-H Exchanger) as it is commonly referred to, is not one protein but consists of a large and growing family of isoforms. These isoforms are derived from distinct genes and play a multiplicity of roles in regulating cellular and organ function in health and disease. To date, eight NHE isoforms, termed NHE-1 to NHE-8, have been cloned. While most NHE isoforms demonstrate substantial tissue specificity, NHE-1 is ubiquitously expressed in virtually all tissues and has been generally referred to as the housekeeping isoform. Other unique sodium-hydrogen exchangers have also been described, such as the newly identified colonic chloride-dependent NHE or the NhaA of E. coli and other species.

This volume brings together international authorities to review major advances in distinct areas of NHE research. These state-of-the-art reviews address a broad range of complementary topics, progressing from the structure and regulation of NHEs to targeting NHE as a therapeutic modality for the treatment of pathological conditions. Indeed, the past two decades have seen startling and rapid advances in our understanding of the regulation of the activity of many NHE isoforms, as well as in the identification and cloning of novel isoforms. NHE has been directly implicated in several pathologies, most notably in the damage that occurs to the myocardium during ischemia and reperfusion and in cardiac hypertrophy and failure. In the area of therapeutics, chemical synthesis of isoform-selective NHE inhibitors has led to the initiation of a number of clinical trials, particularly in the area of cardiovascular disease. Emerging evidence indicates that targeting NHE may also hold promise for other conditions, as discussed in this volume.

The chapters that comprise this volume address the basic structure, function and regulation of NHE proteins, their roles in various diseases, and the development, characterization and clinical evaluation of NHE inhibitors. By covering such a broad range of complementary topics, from molecular biology to clinical therapeutics, this unique volume provides an opportunity for students, basic scientists and clinicians to learn the newest developments in this rapidly evolving field.

We thank all those who helped bring this book to fruition, in particular Pamela Burgess for her outstanding word processing skills. Ultimate thanks go to all contributors without whom this book would not have been possible.

Morris Karmazyn (London, Ontario, Canada)
Metin Avkiran (London, United Kingdom)
Larry Fliegel (Edmonton, Alberta, Canada)

Chapter 1

REGULATION OF INTRACELLULAR pH IN MAMMALIAN CELLS

Kenneth W. Spitzer[1] and Richard D. Vaughan-Jones[2]
[1]Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah, Salt Lake City, UT, USA and [2]Burdon Sanderson Cardiac Science Centre, University Laboratory of Physiology, Parks Road, Oxford, UK

1. INTRODUCTION

The extra- and intracellular concentration of free H^+ ions is one of the most tightly controlled parameters in mammals. For example, the range of arterial pH values compatible with life in humans is approximately 6.9 to 7.8, which corresponds to hydrogen ion concentrations of only 126 and 16 nM, respectively, where $[H^+] = 10^{-pH}$ M. Normal arterial pH is approximately 7.35 to 7.45. Strictly speaking, free H^+ ions exist in body fluids combined with water, mainly as hydronium ions (H_3O^+). Despite their low concentration in most body compartments, ~nM levels compared to mM levels for Na^+ and K^+, H^+ ions have striking effects on a wide variety of biological processes. These effects are facilitated by the high reactivity of H^+ ions and the abundance of H^+ binding sites on macromolecules and metabolites.

Variations in ambient pH alter the occupancy of acidic and basic groups on proteins and other molecules. The resulting change in net charge affects interactions with other molecules and the distribution of intra-molecular charge. Thus the acid-base status of the extra- and intracellular milieu influences numerous cellular properties, including the conformational state of proteins, ligand-receptor interactions, transmembrane flux of K^+, Na^+, Cl^- and Ca^{2+} via channels and transporters, enzyme activity, Ca^{2+} binding to

molecules, and transmembrane distribution of charged molecules including drugs.

Despite constant production of volatile (H_2CO_3) and nonvolatile acids, stable but different pH values are maintained in the extra- and intracellular compartments, including the cytoplasm and intracellular organelles. Plasma pH is maintained by the combined actions of the lungs to eliminate CO_2 and the kidneys to regulate HCO_3^- reabsorption. At the cellular level, several ion transport systems operate to maintain steady-state intracellular pH (pH_i) and to restore pH_i following acute acid-base challenges. Similarly, ion transporters largely control the luminal pH of organelles. This chapter focuses on intracellular pH regulation.

2. INTRACELLULAR COMPARTMENTALIZATION OF pH

Under normal conditions the pH of the cytoplasm in mammalian cells is approximately 7.1 to 7.3. However, there is considerable heterogeneity in the luminal pH of organelles within this compartment. The lysosomes are the most acidic with a pH of 4.6 to 5.0 (1). Endosomal pH is somewhat higher at 5 to 6 (1) and Golgi pH is even higher at ~ 6.5 (2,3). The pH of the nucleus (4) and endoplasmic reticulum (2) are reported to be the same as that of the cytoplasm and the highest organellar pH values (~8.0) occur in the mitochondria (3,4).

3. CYTOPLASMIC pH

To appreciate the importance of ion transport in controlling cytoplasmic pH, it is useful to compare the normal steady-state pH_i of approximately 7.2 to that predicted assuming H^+ ions are membrane permeable and passively distributed across the plasmalemma. In this situation membrane potential (V_m) and the extracellular pH (pH_o) are the only determinants of pH_i and the H^+ equilibrium potential (E_H) is equal to V_m, with $E_H = 2.3RT/zF(pH_i-pH_o)$ where z, R, T and F have their usual meanings. For a pH_o of 7.4 and a V_m of –80mV this yields a pH_i of approximately 6.0 at 37°C. Thus normal cells have a much lower $[H^+]_i$ than predicted from electrochemical equilibrium. This means that under normal steady-state conditions H^+ ions are transported out of cells or their base equivalent is transported into cells against the prevailing electrochemical gradient.

4. INTRACELLULAR BUFFERS

Intracellular buffers act to attenuate changes in pH_i elicited by addition or removal of H^+. Thus they provide the cell's first line of defense against the adverse effects of excessive fluctuations in pH_i. Buffering occurs with a rapid time course and the quantity of acid (or base) bound or released by buffers can be considerable. Buffers will not, however, restore pH_i to its original value following an acid-base perturbation. This requires ion transporters that operate with a slower time course (mins).

Several excellent discussions of pH buffering are available (5-9). Three types of intracellular buffering mechanisms are present in mammalian cells: physicochemical, biochemical and organellar. Physicochemical buffering is provided by a variety of weak acids and bases that rapidly interact with H^+ (and OH^-) according to the reaction: $HA \leftrightarrows A^- + H^+$. Biochemical buffering is mediated by H^+ consumption (or production) via metabolic pathways and organellar buffering involves uptake and release of acid equivalents by organelles. The contributions of biochemical and organellar buffering to acute acid or base loads is not completely resolved but is likely to be less than that provided by physicochemical buffering. The presence of buffers means that very few cytoplasmic H^+ ions are not reversibly bound and make a contribution to the measured pH_i.

Total intracellular buffering is provided by the sum of intrinsic buffers (i.e., those not involving the CO_2/HCO_3^- buffer system) and CO_2-dependent buffering. Although the detailed composition of intrinsic buffers is not resolved for all mammalian tissues, a high percentage of buffering in skeletal and heart muscle is provided by histidine residues on proteins, histidine-containing peptides (e.g., carnosine, anserine, homocarnosine), inorganic phosphate and taurine (10,11). Since the intrinsic buffers are largely confined to the cytoplasm and their concentration remains constant, they operate as a closed buffer system.

In contrast, CO_2-dependent buffering operates as an open buffer system according to the reversible CO_2 hydration reaction:

$$CO_2 + H_2O \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO_3^- \qquad (1)$$

Under normal steady-state conditions the partial pressure of CO_2 in plasma (PCO_2) remains constant via alveolar ventilation or in the case of a cell bath by constantly superfusing the cells with a HCO_3^--containing solution equilibrated with CO_2. Assuming the cell membrane is permeable to CO_2 and that PCO_2 is the same inside and outside the cell, then with pH_o=7.4, pH_i=7.1 and $[HCO_3^-]_o$=24 mM, the calculated $[HCO_3^-]_i$ is 12 mM, where: $[HCO_3^-]_i = [HCO_3^-]_o \times 10^{(pH_i - pH_o)}$.

The addition of H^+ ions to the cytoplasm drives reaction 1 to the left forming CO_2, which diffuses out of the cell and either enters the blood or the superfusate and is swept away. The maintenance of a constant external PCO_2 helps promote H^+ buffering by preventing CO_2 accumulation. However, excessive acidity can reduce $[HCO_3^-]_i$ to very low levels. The addition of base drives the reaction to the right, promoting the formation of $[HCO_3^-]_i$ which can increase to very high levels due to the constant source of CO_2. The speed of the forward and reverse hydration reactions requires many seconds even in the presence of carbonic anhydrase which catalyzes this reaction (12). Thus the buffering action of CO_2/HCO_3^- is slower than that of intrinsic buffers.

Buffering is quantified as buffering power (β, mmol pH^{-1} $liter^{-1}$) and is defined as: moles of added acid or base divided by the resulting change in pH. Because of their multi-component nature, the total buffering power of intrinsic buffers (β_i) must be determined experimentally (5,8). Intracellular CO_2-dependent buffering (βCO_2) can also be experimentally measured or calculated as: 2.3 $[HCO_3^-]_i$, provided equilibrium conditions prevail. If the intracellular CO_2/HCO_3^- buffer system has not equilibrated, its contribution to total intracellular buffering will be attenuated. Under these conditions, estimating transmembrane flux of acid-equivalents from the measured rate of change of pH_i becomes very complex (13). Total buffering power (β_T) is equal to the sum of β_i and βCO_2. An important characteristic of buffers is the pH dependence of their buffering power (Figure 1). Thus, the relative contribution of βCO_2 and β_i to β_T changes as pH_i is varied.

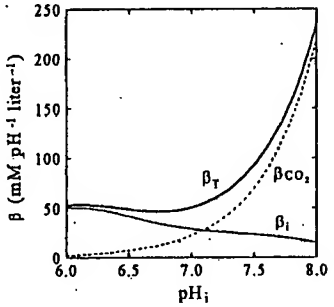

Figure 1. Relationship of β_i, βCO_2 and β_T to pH_i. βCO_2 was calculated as 2.3 $[HCO_3^-]_i$ assuming pH_o=7.4 and $[HCO_3^-]_o$=24 mM. The curve labeled β_i is the best fit of measurements from resting guinea-pig pig ventricular myocytes (14). $\beta_T = \beta_i + \beta CO_2$.

5. MEASUREMENT OF pH_i DURING ACID-BASE LOADING

Several techniques are available to measure pH_i: pH-sensitive electrodes (15), nuclear magnetic resonance (16) and pH-sensitive fluorescent dyes (17), including BCECF (18) and carboxy-SNARF-1 (19,20). The fluorophores are frequently used for single cells and cultured cell monolayers because of the ease of introducing their esterified forms into the cytoplasm, their high H^+ sensitivity, rapid kinetics and ease of calibration.

The transporters that regulate pH_i (acid extruders and acid loaders) can be activated by rapidly introducing acid or base into cells. For example, the ammonium prepulse technique (21) is widely used for acid loading (Figure 2A). The initial alkalosis results from influx of the highly permeant weak base, ammonia (NH_3). pH_i subsequently falls during the NH_4Cl pulse partly because NH_4^+ enters the cell and dissociates, releasing H^+. On removal of NH_4Cl an intracellular acid load is created as all of the NH_4^+ exits the cell in the form of NH_3, causing intracellular retention of H^+. Acid extruders mediate the subsequent recovery of pH_i.

Similarly, a weak acid (e.g., acetic acid) prepulse can be used to create an intracellular alkaline load (8,14,22) (Figure 2B). Uncharged CH_3COOH rapidly enters the cell and dissociates, releasing CH_3COO^- and H^+ causing pH_i to fall. If net acid extrusion occurs during the acetate pulse, as it will if acid extruders are operating, then pH_i will overshoot its initial value upon removal of acetate, creating an intracellular alkalosis as CH_3COO^- and H^+ recombine and exit the cell. Acid loaders mediate the subsequent recovery of pH_i.

Because of the presence of intracellular buffers, the net rate of acid extrusion or acid loading via transporters or channels cannot be determined solely from instantaneous dpH_i/dt but is equal to: $dpH_i/dt \times \beta_T$ (units, mEq $liter^{-1}$ min^{-1}) or $dpH_i/dt \times \beta_T \times$ volume/surface (units, mEq $liter^{-1}$ min^{-1} cm^{-2}). CO_2/HCO_3^- is typically removed from the superfusate during *in vitro* experiments designed to inhibit HCO_3^--dependent pH_i regulatory systems (e.g., Figure 2). In this setting βCO_2 is usually negligible and $\beta_T = \beta_i$.

6. TRANSPORT SYSTEMS THAT REGULATE pH_i

Both acid extruders and acid loaders are required to maintain normal steady-state pH_i and to restore pH_i following intracellular acid and alkaline loading. This provides an integrated control system for rapidly responding to

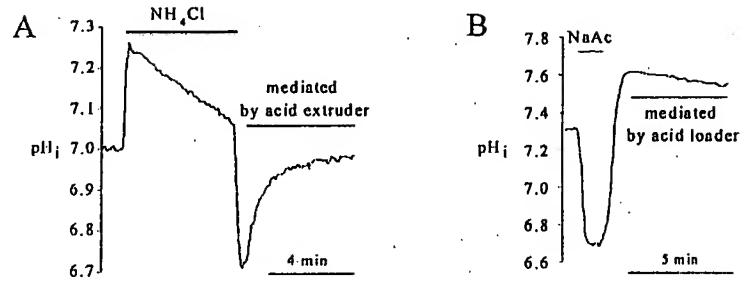

Figure 2. NH_4Cl prepulse (10mM) applied to a ventricular myocyte to induce intracellular acidosis and activate acid extruders. *B.* NaAc prepulse (40mM, substituted for NaCl) applied to a ventricular myocyte to elicit intracellular alkalosis and activate acid loaders. Bathing solutions for both cells contained no added CO_2/HCO_3^- (pH_o 7.4).

deviations in pH_i from its normal set-point of approximately 7.2. Transport activity is low at the set-point, with acid extruders increasingly activated by a fall in pH_i and loaders activated by a rise. The relative contribution of each transporter to pH_i control varies considerably from one cell type to another. The principal acid extruders include: Na^+-H^+ exchanger (NHE), Na^+-HCO_3^- cotransporter (NBC), Na^+-dependent Cl^--HCO_3^- exchanger (NDCBE) and ATP-dependent H^+ pump (H^+-ATPase). The main acid loaders are Cl^--HCO_3^- exchanger (anion exchanger, AE) and, at least in cardiac cells, Cl^--OH^- exchanger (CHE). The proton-linked monocarboxylic acid transporter (MCT) translocates solutes such as lactic acid across the plasmalemma of many cell types, and thus also influences pH_i. Physiologically, it can act as either an acid loader or extruder depending on the prevailing transmembrane H^+ and lactate$^-$ ion gradients. Except for H^+-ATPase, none of the carriers has a direct requirement for ATP hydrolysis to mediate ion movement.

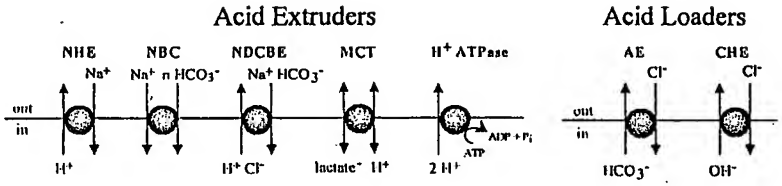

Figure 3. Transporters that regulate mammalian intracellular pH.

6.1 NHE

As discussed by Orlowski and Grinstein in the following chapter, the NHE family of transporters includes at least eight isoforms (NHE1-8) of which NHE1 is the most widely expressed and provides the principal pathway for acid extrusion in most mammalian cells (23-31). The following comments are restricted to NHE1. The transporter is electroneutral with a stoichiometry of 1:1. An important consequence of electroneutrality is that the direction of transport is determined by the energy available in the chemical gradients for Na^+ and H^+, which are inwardly and outwardly directed, respectively. NHE could, in theory, drive pH_i to an equilibrium value of approximately 8 in most cell types under normal steady-state conditions if its activity were determined solely by the magnitude of the transmembrane Na^+ and H^+ ion gradients. However, the presence of an internal H^+ modifier site slows acid extrusion to low levels when pH_i reaches approximately 7.1, while more acidic values markedly stimulate H^+ efflux (32). In contrast, low pH_o reduces H^+ efflux. NHE is inhibited by amiloride and its derivatives (33) and by benzoyl guanidinium compounds such as HOE642 (cariporide) (34).

An example of the steep dependence of NHE-mediated acid extrusion on pH_i is shown in Figure 4. In many cell types, this H^+ efflux versus pH_i curve is shifted in the alkaline direction by a variety of agents, including angiotensin, endothelin, thrombin, and catecholamines by activating receptor-mediated signaling pathways that alter the phosphorylation state of the transporter or possibly regulatory accessory proteins. ATP and hyperosmotic stress have a similar effect on the curve, mediated through what appears to be phosphorylation-independent mechanisms. In all cases, the right shifted efflux-pH_i curve results in a more rapid rate of pH_i recovery from intracellular acidosis and an increased steady-state pH_i.

In addition to its effect on H^+ efflux, NHE activation also increases $[Na^+]_i$ which, in heart muscle, can elevate $[Ca^{2+}]_i$ via effects on sarcolemmal Na^+-Ca^{2+} exchange. Interestingly, this promotes recovery of contraction during sustained acidosis (35,36). However, the excessive activation of cardiac NHE that accompanies postischemic reperfusion can cause abnormally high $[Ca^{2+}]_i$, resulting in myocardial injury and arrhythmias (37). Inhibition of NHE minimizes these effects (34). Further discussion of the importance of NHE to ischemic and reperfusion injury in the heart can be found in Chapter 15 and Chapters 17 to 20 of this volume.

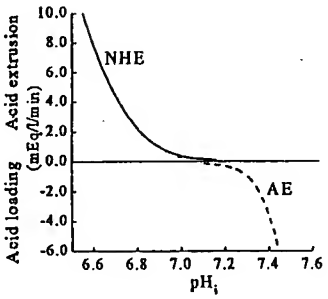

Figure 4. Effect of pH_i on H^+ extrusion via NHE in ventricular cells (pH_o 7.4). Also shown is the response of AE to pH_i. Best fit of results from Leem et al (14).

6.2 NBC

In addition to NHE, many cell types also rely on HCO_3^- influx via a Na^+-HCO_3^- cotransporter as an acid extrusion pathway. Transport activity does not involve Cl^-. Several reviews of HCO_3^--dependent pH_i regulatory systems, including NBCs, are available (38-41). NBCs are members of the SLC4 (solute carrier) bicarbonate transporter gene family, which also includes AE and NDCBE (42). The isoforms of human NBC cloned to date include NBC 1-4 (39).

Compared to NHE, less is known concerning the tissue distribution of NBC, its role in pH_i regulation, substrate specificity and modulation by signaling pathways. Molecular biological studies and functional analyses have revealed both electroneutral and electrogenic forms of NBC in which 1 Na^+ ion is cotransported with either 1 or 2 and 3 HCO_3^- ions, respectively. NBCs with 1:1 and 1:2 stoichiometries normally act as acid extruders (HCO_3^- influx) and have been found in a variety of cell types, including heart (40,43,44), liver (44,45), colon (46) and brain (47). Intracellular acidosis activates these NBCs and promotes acid extrusion, whereas low pH_o inhibits acid efflux. At acidic values of pH_i the rate of acid extrusion via NBC is typically less than that mediated by NHE. In contrast to NBCs with 1:1 and 1:2 stoichiometries, those with 1:3 coupling usually mediate Na^+ and HCO_3^- efflux as occurs in proximal tubule cells of the kidney (48). NBC activity is inhibited by the stilbene derivative DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonate) (49).

In contrast to the beneficial role of NBC in mediating acid extrusion, recent evidence from heart muscle indicates that the accompanying Na^+ influx may contribute to postischemic reperfusion injury by promoting $[Ca^{2+}]_i$ overload (50).

6.3 NDCBE

This acid extruder is electroneutral and links sodium and bicarbonate influx with chloride efflux. Transport activity is blocked in the absence of internal Cl^-. The exact composition of the acid equivalents transported is unresolved. For example, the scheme in Figure 3 showing $1Na^+$-$1HCO_3^-$ exchanged for $1Cl^-$-$1H^+$ is equivalent to $1Na^+$-$2HCO_3^-$ exchanged for $1Cl^-$. NDCBE is activated by intracellular acidosis, blocked by stilbenes (DIDS and SITS) and is found in several cell types, including nerve (51) and aortic endothelial cells (52).

6.4 MCT

The primary function of this family of transporters is reversible transmembrane movement of monocarboxylates such as lactate, pyruvate, acetate, and ketone bodies that are produced and utilized by several metabolic pathways (53,54). Seven mammalian isoforms have been identified to date, designated MCT1-7 (55). MCTs cotransport monocarboxylate ions and protons with a 1:1 stoichiometry and thus are electroneutral. The contribution of MCT activity to pH_i regulation is best characterized in heart and skeletal muscle where the transporter mediates net H^+ efflux when intracellular lactic acid concentration increases (54,56). Transport activity is inhibited by several agents, including cyanocinnamate derivatives and stilbene disulfonates (53,54).

6.5 H^+-ATPase

Since this electrogenic transporter is located in the membrane of several intracellular compartments, including endosomes, lysosomes and phagosomes it is called the vacuolar H^+-ATPase, or V-ATPase (57,58). In this setting it functions as an acid loader, helping to maintain the low luminal pH required for a variety of functions. However, in some cell types, including macrophages (59) and tumor cells (60) the transporter is located in the plasma membrane where it functions as an acid extruder ($2H^+$ out, no counter-ion flux) and helps regulate pH_i. V-ATPases are inhibited by bafilomycins (61).

6.6 AE and CHE

Both of these acid loaders are electroneutral. AE is widely distributed and represented by two gene families SLC4 and SLC26 (42). The best-characterized sodium-independent anion exchangers (AE1-3) are members of the SLC4 family and mediate Cl^--HCO_3^- exchange, typified by the classical AE1 in erythrocytes (42,62). Several members of the SLC26 family have also been shown to function as Cl^--HCO_3^- exchangers (42). CHE has been functionally characterized only in heart muscle and its molecular properties are unresolved (22,63).

Along with NHE, AE makes a major contribution to pH_i control in many tissue and cell types, including neutrophils (64), heart (22,65), smooth muscle (66), vascular endothelial cells (67), and liver (68). The direction of exchange is determined by the relative magnitudes of the transmembrane Cl^- and HCO_3^- gradients. Since the Cl^- gradient is normally several times greater than that of HCO_3^-, bicarbonate efflux (acid-equivalent influx) is energetically favored under normal conditions. Intracellular alkalosis stimulates HCO_3^- efflux via AE (Figure 4), which helps return pH_i to its normal steady-state value. In some cell types extracellular ATP stimulates AE1 via a tyrosine kinase pathway, which has the effect of decreasing steady-state pH_i and increasing the rate of recovery from intracellular alkalosis (69,70). AEs are inhibited by stilbene disulfonates (49).

6.7 Other Acid Extruders And Loaders

There are additional transporters not illustrated in Figure 3 that affect pH_i. They include: a) the acid extruder, H^+, K^+ ATPase, which catalyzes electoneutral H^+ efflux for K^+ uptake in several cell types, including the stomach and kidney (71) and b) two acid loaders, K^+-HCO_3^- cotransporter (HCO_3^- efflux) found in the kidney (72), and the plasma membrane Ca^{2+}-ATPase which catalyzes Ca^{2+} efflux for H^+ uptake and is found in several cell types, including erythrocytes (73), smooth muscle (74) and neurons (75,76).

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Chapter 2

MOLECULAR AND FUNCTIONAL DIVERSITY OF MAMMALIAN Na^+/H^+ EXCHANGERS

John Orlowski‡ and Sergio Grinstein*
*‡Department of Physiology, McGill University, Montreal, Quebec, Canada: *Programme in Cell Biology, The Hospital for Sick Children Research Institute, Toronto, Ontario, Canada*

1. INTRODUCTION

Excess acid produced by cellular metabolism must be buffered or actively extruded in order to maintain acid-base equilibrium and proper cell function. In mammalian as well as other eukaryotic cells, restoration of steady-state pH following acidification is achieved most efficiently by the electroneutral exchange of intracellular H^+ for extracellular Na^+; a process that is mediated by a family of integral membrane cation transporters commonly referred to as Na^+/H^+ antiporters or exchangers (NHE). Aside from cytoplasmic pH homeostasis, the NHEs contribute to a spectrum of other physiological processes, including cell volume regulation, fluid secretion, and salt and water absorption across epithelia (1-4). Additionally, NHE activity is thought to influence cellular events such as adhesion, migration, proliferation, and apoptosis (5,6). Abnormal activities of certain NHEs are also associated with the progression of several disease states, including essential hypertension (7,8), congenital secretory diarrhea (9), diabetes (10-13), and ischemia/reperfusion-induced injuries to tissues such as heart (14), brain (15) and kidney (16,17). This review is intended to briefly summarize recent findings regarding the molecular and functional heterogeneity of the mammalian NHE gene family.

2. GENETIC HETEROGENEITY

In mammals, eight distinct NHE genes (NHE1 to NHE8) have been described to date that are dispersed throughout the genome (18-25) (*Table 1*). Each gene appears to encode a single protein with the exception of NHE6, which in humans undergoes alternative RNA splicing to form at least two gene products designated NHE6-0 (referring to the original cDNA clone) (23) and NHE6-1 (26). The latter contains an additional 96-nucleotide insert encoding 32 amino acids situated between original residues Leu^{143}-Val^{144} and is predicted to reside in the second exoplasmic loop. Using polymerase chain reaction amplification, we have recently identified a third putative variant of human NHE6 in brain (tentatively named NHE6-2) that also encodes an alternatively-spliced insert in this same position and exhibits ~85% identity to the corresponding segment present in NHE6-1[1]. It is worth noting that these spliced segments share high homology to an analogous region of NHE7. It has yet to be determined whether these NHE6 variants show unique properties, such as tissue-specific expression or altered catalytic behaviour. Overall, the NHEs show wide divergence in their primary structures, varying between 25 to 70% amino acid identity (*Table 2*).

Table 1. Diversity of Mammalian Na^+/H^+ Exchangers

Isoform	Ch[a]	Tissue Distribution[b]	Membrane Location	Principal Function	Murine Null Phenotype
NHE1	1p35	Ubiquitous	plasmalemma (basolateral surface of epithelia)	cytoplasmic pH & cell volume	ataxia, seizures, postnatal lethal
NHE2	2q11	GI > SkM >>> Kd, Br, Ut, T >> Ht, Lg	plasmalemma (apical surface of epithelia)	fluid secretion	↓ viability of gastric parietal cells; ↓ parotid gland fluid secretion
NHE3	5p15	GI > Kd >>>>Br (other epithelia)	plasmalemma (apical surface of epithelia) & recycling	Na^+ & HCO_3^- (re)absorption; endosomal acidification	diarrhea, acidotic, hypotensive

[1] M. Numata, I. Virdee and J. Orlowski; *unpublished data*

Isoform	Ch[a]	Tissue Distribution[b]	Membrane Location	Principal Function	Murine Null Phenotype
			endosomes		
NHE4	2q12-34	St >>>> Kd, Br	plasmalemma (basolateral surface of epithelia)	cytoplasmic pH & cell volume?	?
NHE5	16q22	Br (neurons)	plasmalemma & recycling endosomes (synaptic vesicles)	endosomal pH?	?
NHE6	Xq26	Ubiquitous	endosomes	endosomal pH?; protein sorting?	?
NHE7	Xp11	Ubiquitous	*trans*-Golgi network & endosomes	organellar pH?; protein sorting?	?
NHE8	20q13	Ubiquitous	plasmalemma?	?	?

[a] Chromosomal (Ch) location of human NHE genes.
[b] Br, brain; GI, gastrointestinal tract; Ht, heart; Kd, kidney; Lg, lung; SkM, skeletal muscle, T, testis; Ut, uterus

Table 2. Similarity of Mammalian Na^+/H^+ Exchangers

	hNHE1	hNHE2	hNHE3	rNHE4	hNHE5	hNHE6	hNHE7	hNHE8
hNHE1	100	46	39	39	40	22	25	20
hNHE2	46	100	42	54	42	24	25	20
hNHE3	38	41	100	35	55	24	26	21
rNHE4	44	62	41	100	39	26	26	24
hNHE5	37	38	51	31	100	21	23	20
hNHE6	27	29	30	28	28	100	71	29
hNHE7	28	28	29	25	29	65	100	28
hNHE8	29	28	30	30	28	33	35	100

Pairwise comparison of the percent similarity of human (h) or rat (r) Na^+/H^+ exchanger isoforms. The values were calculated by dividing the number of identical matches between each pair by the length of the corresponding isoform listed in the leftmost column. This matrix yields two values for each pair of isoforms. The overall percent similarity between any pair was calculated as the average of these two values. The lengths of NHE1, -2,-3, -4, -5, -6, -7, -8 are 815, 812, 834, 717, 896, 669, 725, 581 amino acids, respectively.

2.1 Structure And Function Relations

Despite their sequence heterogeneity, a similar membrane topology can be predicted for all isoforms, with 12 membrane-spanning segments (M) at the *N*-terminus that are relatively conserved amongst the isoforms and a large hydrophilic region at the *C*-terminus that is considerably more divergent and located in the cytoplasm. Recent topological mapping studies of NHE1 using intact cells have largely corroborated this model with some refinements (27,28) (illustrated in *Figure 1*). Specifically, by testing the accessibility of single cysteine substitutions of a cysteine-less mutant of NHE1 to the membrane-impermeant thiol-modifying reagent biotin maleimide in both intact and permeabilized cells, Wakabayashi and

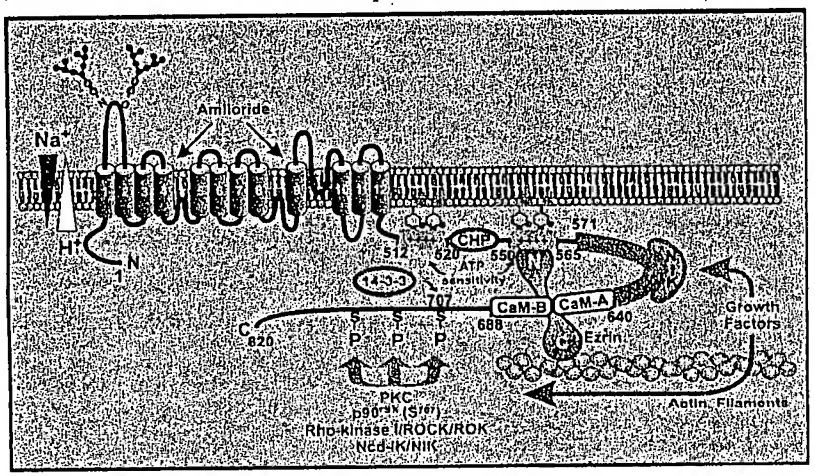

Figure 1. Structural Organization and Regulation of the Na^+/H^+ Exchanger NHE1 Isoform. The primary structure is numbered according to the rat sequence.

colleagues (28) proposed a model whereby the earlier predicted M10 and adjacent intracellular loop 5 (IL5) segments form instead a large re-entrant loop (R-loop) structure following M9, and that the predicted M11 and extracellular loop 6 (EL6) segments now constitute M10 and M11, respectively. This R-loop resembles the pore- or P-loop structures that line the ion conduction pathway of ion channels and pumps (29), and may perform a similar function in the NHEs. In addition, cysteines placed within the predicted IL2 and IL4 segments were also accessible to extracellularly-applied biotin maleimide, suggesting that these loops may invaginate into the lipid bilayer and face an aqueous environment, possibly forming part of the ion translocation pathway. In contrast to NHE1, another study using an *in*

vitro transcription/translation assay defined only 11 membrane-spanning segments in NHE3 due to the presence of a cleaved N-terminal signal peptide (30). Whether this cleavage actually occurs *in vivo* is unknown.

In addition to the above, immunological studies have provided some indication that certain regions of the cytoplasmic C-terminus of NHE1 (31) and NHE3 (32) may be exposed extracellularly, despite the absence of obvious conventional α-helices capable of traversing the membrane twice in order to maintain the extreme C-terminus in the cytoplasm. Less is known about their tertiary or quaternary structure, although recent evidence suggests that they exist in the membrane as homodimers (33,34). Certain NHEs (*i.e.*, NHE1, NHE2, and NHE6) are also glycosylated, although the functional significance of this posttranslational modification is not apparent (26,35,36).

Biochemical and mutational analyses indicate that the *N*-terminus is responsible for ion translocation (37) and drug recognition (38-42), with the latter involving crucial residues within M4 and M9. By contrast, the *C*-terminal region is chiefly involved in isoform-specific regulation by growth factors and other mitogens, consistent with its cytoplasmic orientation (37,43-47).

Recent molecular studies indicate that both direct phosphorylation of serine residues and phosphorylation-independent events are required to elicit responsiveness of the NHEs to various stimuli. This is best exemplified by NHE1, which has been studied more extensively than other isoforms. For instance, numerous growth factors and phorbol esters that activate NHE1 are thought to transduce their signals through the MEK-ERK-$p90^{rsk}$ pathway, since $p90^{rsk}$ can directly phosphorylate S^{707} of rat NHE1, which then is capable of binding the multifunctional scaffolding protein 14-3-3 (48,49). NHE1 is also a substrate for other kinases, including p160 Rho-associated kinase (ROCK) which mediates signals from integrin receptors (50),and Nck-interacting kinase (NIK) which transduces signals from receptor tyrosine kinases independently of the ERK-$p90^{rsk}$ pathway. However, the mechanism by which protein phosphorylation leads to accelerated transport remains unknown.

Aside from direct phosphorylation, regulation of NHE1 also involves the participation of other ancillary regulatory factors, some of which may also be potential targets of protein kinases. Ca^{2+}/calmodulin directly binds and activates the NHE1 isoform and likely contributes to the Ca^{2+} regulation of this isoform (51,52). Two other Ca^{2+}-binding proteins that share ~50 identity, called calcineurin B homologue protein-1 and -2 (CHP1 and CHP2), also interact with the juxtamembrane region of the C-terminus of NHE1, and their association with NHE1 appears to be critical for transport activity (53,54). Of particular note is the ability of CHP2 to constitutively activate NHE1 and raise the steady-state intracellular pH which seemingly

protects cells from serum deprivation-induced death. The mechanistic basis for this phenomenon however remains unknown. In addition, two positively-charged clusters that flank the CHP-interacting region bind phosphatidylinositol 4,5-bisphosphate (PIP_2) *in vitro* and appear to be critical for basal NHE1 activity (55). Mutation of both sites greatly reduces NHE1 activity and its sensitivity to ATP. The distal site can also associate with the cytoskeletal-associated proteins ezrin, radixin and moesin (ERMs) *in vitro* and *in vivo* (56). The binding of NHE1 to the ERMs is proposed to play an important role in regulating the cortical cytoskeleton and cell shape independently of cation translocation (56).

2.2 Tissue Expression, Subcellular Distribution, And Physiological Roles

In addition to their molecular heterogeneity, the NHEs show considerable diversity in their patterns of tissue expression, membrane localization, and functional roles (summarized in *Table 2-1*). Of these, five (NHE1-5) are principally active in the plasma membrane, whereas two (NHE6 and NHE7) reside predominantly within intracellular compartments. The subcellular distribution of the newly discovered NHE8 is uncertain and awaits further experimentation.

NHE1 is ubiquitously expressed and is noted for its high sensitivity to inhibitory drugs such as amiloride and benzoylguanidiumium derivatives. It is chiefly responsible for restoration of steady-state pH_i following cytosolic acidification and for maintenance of cell volume. Recent findings indicate that it is also crucial for organismal function and survival. Mice with null mutations of *Nhe1* exhibit locomotor abnormalities, epileptic-like seizures, and considerable mortality (67%) prior to weaning (57,58). These changes are associated with selective loss of neurons in the cerebellum and brainstem (57). Moreover, hippocampal CA1 neurons isolated from these animals display enhanced membrane excitability and increased Na^+ current density, implicating a functional association between NHE1 and voltage-sensitive Na^+ channels that seemingly contributes to neural dysfunction when disrupted (59). However, the precise molecular nature of this relationship is unknown.

In contrast to the loss of NHE1 function, hyperactivation of NHE1 is thought to contribute to the progression of cardio- and cerebro-vascular injuries following episodes of ischemia and reperfusion. Moderate to severe reductions in blood flow significantly decrease the supply of glucose and oxygen required to maintain the high energy demands of the heart and brain. As a consequence, ATP stores are rapidly depleted, and lactate, pyruvate and protons accumulate due to anaerobic metabolism of the limited stores of glucose and glycogen (reviewed in Refs. (60-62)). This results in rapid

increases in both intracellular and extracellular acidity which disrupts the homeostasis of other ions; most notable are the excess accumulation of intracellular Na^+ (via the Na^+/H^+ exchanger) and Ca^{2+} (via the Na^+/Ca^{2+} exchanger acting in reverse mode) (63-65). This, in turn, precipitates a series of other cellular changes that lead to tissue dysfunction and ultimately tissue damage, including free radical toxicity, cellular edema, apoptosis and necrosis (66,67). A role for NHE1 is most convincingly demonstrated by numerous studies using specific NHE antagonists, including various amiloride analogues (14,68,69), and the more selective NHE1 inhibitors such as the benzoylguanidine compounds (*e.g.*, HOE694, HOE642 (cariporide), and EMD85131) (70-77) and SM-20220 (15,78) that significantly reduced Na^+_i and Ca^{2+} overloads and effectively prevent cardiac and neural injuries associated with ischemia both *in vitro* and *in vivo*. Further discussion of these issues can be found in Chapter 12 which is related to the role of NHE1 in damage to the central nervous system as well as in Chapters 15 and 17-20 which deal with myocardial aspects of NHE1.

By comparison, NHE2-4 are predominantly expressed in epithelia of the kidney and gastrointestinal tract (1), but are also detected at low levels in discrete regions of the brain (79). In polarized epithelia, NHE4 is located at the basolateral membrane (80-82). While the cellular role of NHE4 has yet to be ascertained, it may function in a similar manner to NHE1, particularly in the macular densa (81) and intercalated cells of the cortical collecting duct (83,84) where basolateral Na^+/H^+ exchange activities have been measured but lack NHE1 expression.

In contrast, NHE2 and NHE3 reside along the apical membranes of discrete nephron and intestinal segments (81,85,86). In kidney, NHE2 is predominately found in the cortical thick ascending limb, macula densa, distal convoluted tubules and connecting tubules (81,87), whereas NHE3 is located predominantly in the proximal tubule and to a lesser extent in the medullary thick ascending limb (85,88). In addition to the microvillus, NHE3 (89,90), but not NHE2 (91), is detected in a discrete population of clathrin-associated recycling vesicles, where it could serve as a regulated reservoir of functional transporters. Several lines of evidence indicate that NHE3 is the major contributor to bulk Na^+ and fluid reabsorption by the proximal tubule (3,92-94). The associated secretion of H^+ by NHE3 into the lumen of renal tubules is also essential for approximately two-thirds of renal HCO_3^- reabsorption (92,95-97). Proton secretion by NHE2 also regulates HCO_3^- reabsorption in the distal convoluted tubule, although its contribution to plasma HCO_3^- levels is less evident (98). NHE3, but not NHE2, was found to regulate basal as well as meal-stimulated ileal Na^+ absorption *in vivo* (3,99). However, the physiological distinction between these two apical transporters is best revealed by studies of NHE2- and NHE3-null mice. Complete disruption of NHE3 function caused slight diarrhea and

alkalinization of the intestinal luminal contents, sharply decreased HCO_3^- and fluid absorption in proximal convoluted tubules, mild acidosis, reduced blood pressure, elevated serum aldosterone and higher renal renin mRNA expression, consistent with the animals being in a volume-contracted state (94,100). These effects differ considerably from those observed for knockout mice lacking the NHE2 gene, which show severe degeneration of gastric parietal and zymogenic cells and significantly decreased parotid gland fluid secretion, but no apparent intestinal or renal absorptive defects (100-102). Taken together, these studies highlight the central importance of NHE3 in absorptive functions that profoundly influence systemic electrolyte, acid-base, and blood pressure homeostasis, whereas NHE2 appears to function primarily in secretory processes of certain glands.

Aside from its role in renal and intestinal physiology, there are some indications that NHE3 may also participate in functions of the central nervous system. In brain, NHE3 is detected in cerebellar Purkinje and glial cells (79), but also in chemosensitive neurons of the ventrolateral medulla oblongata that modulate the rate of respiration (103). It's presence in the latter neurons is particularly intriguing in light of recent studies showing that relatively selective antagonists of NHE3 acidify and activate these neurons *in vitro* (103,104) and elevate the central respiratory response to hypercapnia *in vivo* (105); implicating a regulatory role for this isoform in the control of breathing rhythm. On the other hand, mice with targeted disruptions of the *Nhe3* locus do not display obvious neurological symptoms, although effects on respiration were not examined directly (94). Hence, its importance in nervous system function remains to be established.

NHE5 shows the most limited tissue distribution of all NHEs, being largely restricted to neuronal-enriched regions of brains from both rats (106) and humans (22). While NHE5 transcripts have been detected in human spleen and testis, further examination showed that they were either incompletely processed or aberrantly spliced and unlikely to yield functional transporters (22). Structurally, NHE5 is most closely related to NHE3 (~50% amino acid identity) and shares similar pharmacological properties (106,107); namely a low affinity for drugs such as amiloride and benzoylguanidine derivatives. This suggests that NHE5 may be the amiloride-resistant Na^+/H^+ exchanger previously described in hippocampal neurons (108). At present, the subcellular distribution of native NHE5 is unknown and awaits the development of isoform-specific antibodies. However, an epitope-tagged form of NHE5 was shown to accumulate both in the plasma membrane and in transferrin receptor-associated recycling endosomes when ectopically expressed in Chinese hamster ovary (CHO) cells (109). An analogous distribution was also observed in transiently transfected nerve growth factor-differentiated neuroendocrine PC12 cells and primary cultures of rat hippocampal neurons, where NHE5 concentrated

in somatodendritic vesicles, but also in varicosities (*i.e.*, regions enriched in synaptic or synaptic-like microvesicles) along the axons or neurite processes (109).

At present, the physiological role of NHE5 in neuronal function is unknown. However, the tentative morphological localization of NHE5 to synaptic vesicles or synaptic-like microvesicles suggests that NHE5 might play a role in synaptic transmission by functioning in conjunction with the vacuolar H^+-ATPase to acidify the lumen of these compartments. Generation of a proton gradient provides the electrochemical driving force for the uptake of various neurotransmitters (110). As such, vesicular acidification is an important determinant of neurotransmitter concentration, and ultimately of synaptic transmission. The ability of NHEs to regulate acidification of intracellular vesicles is not without precedent. An amiloride-insensitive NHE was found to accelerate the acidification of rat liver early endosomes immediately after their formation (111). Similarly, the epithelial NHE3 isoform increased acidification of recycling endosomes when ectopically expressed in Chinese hamster ovary cells (112). It remains to be seen whether NHE5 is also capable of modulating the luminal acidity of synaptic or synaptic-like vesicles and, if so, whether this has any impact on synaptic transmission. In this regard, it is intriguing to note that genetic mapping studies have localized NHE5 to a region of chromosome 16q22.1 (113) that coincides with a locus linked to a late-onset (20-60 years of age) form of autosomal dominant spinocerebellar ataxia (*SCA4*) (114), implicating NHE5 as a possible candidate gene in the development of this neurodegenerative disease.

NHE6 and NHE7, which are closely related to each other (~ 70% identity), do not accumulate to any significant extent on the plasmalemma and instead localize predominantly to intracellular compartments. Initial transfection studies using HeLa cells suggested that an NHE6-green fluorescent protein chimera localized to mitochondria (23), purportedly accounting for earlier descriptions of a mitochondrial NHE (115). However, other reports (26,116) indicated that both NHE6-0 and NHE6-1 sorted instead to other intracellular compartments, such as recycling endosomes, when transiently overexpressed in cultured mammalian cells. A minor fraction also appeared transiently on the cell surface, although it is unclear whether this occurs normally or is a consequence of heterologous overexpression. Further studies are required to resolve this apparent discrepancy. By comparison, NHE7 is associated with the *trans*-Golgi network and possibly other endosomal vesicles. However, it is unclear whether these vesicles are the same or different from those containing NHE6. Functionally, NHE7 is further distinguished from the plasmalemmal NHEs by its ability to transport either Na^+ or K^+ in exchange for H^+ (24). Since K^+ is the major intracellular cation, it is likely that NHE7 functions

primarily as a K^+/H^+ exchanger. In addition, NHE7 exhibits a high degree of insensitivity to drugs such as amiloride compared to other NHEs, but can be inhibited by benzamil, an amiloride analogue that is a poor antagonist of other NHEs (117). NHE6 may also exhibit similar cation specificities given its close structural relatedness to NHE7, although this has yet to be demonstrated empirically. Orthologs of NHE6 and NHE7 also exist in lower eukaryotes such as *Saccharomyces cerevisiae* (referred to as Nha2 or Nhx1) and *Arabidopsis thaliana* (Nhx1), where they have been implicated in conferring tolerance to high salt environments (118-120) and in the proper sorting of proteins along the secretory pathway (121). In keeping with its mammalian counterparts, a purified histidine-tagged version of *Arabidopsis* NHX1 was shown to catalyze both Na^+ and K^+ transport equally, and to a lesser extent Li^+ and Cs^+, in the presence of a countering H^+ gradient when reconstituted into lipid vesicles. Collectively, these data are consistent with the notion that these transporters function as relatively non-selective monovalent cation exchangers that likely fulfill important roles in the control of organellar pH and volume.

Most recently, another novel NHE-related isoform (NHE8) was cloned from mouse (25) and human[2] that shares only ~25% amino acid identity to all other family members and is ubiquitously expressed. Using a polyclonal anti-NHE8 antibody, Goyal and colleagues showed that NHE8 was present in isolated brush-border membrane vesicles of renal proximal tubule epithelia (25). However, the antibody was ineffective in immuno-cytochemical analyses, precluding precise determination of its subcellular distribution. By contrast, ectopic expression of an epitope-tagged form of human NHE8 in HeLa cells showed a diffuse distribution throughout the cell in a pattern that largely resembled the endoplasmic reticulum, suggesting that NHE8 may be an organellar-type NHE[2]. A more accurate description of its subcellular distribution awaits the development of more versatile NHE8-specific antibodies and dual labelling with organellar markers.

3. CONCLUSION

In summary, the NHE gene family has shown itself to be more diverse that previously anticipated from earlier functional studies. Much progress has been made over the last few years in characterizing the functional and regulatory properties of the plasma membrane-type NHEs. Studies of the organellar-type NHEs are still in their infancy, but undoubtedly will provide exciting new insights into their contributions to cellular physiology.

[2] M. Numata, S. Grinstein and J. Orlowski, *manuscript in preparation*

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Chapter 3

TWO FUNDAMENTAL REGULATORY FACTORS OF THE Na^+/H^+ EXCHANGERS *THE PROTON AND CHP*

Shigeo Wakabayashi, Tianxiang Pang, Takashi Hisamitsu and Munekazu Shigekawa

Department of Molecular Physiology, National Cardiovascular Research Institute, Osaka, Japan

1. INTRODUCTION

The Na^+/H^+ exchangers (NHEs) are plasma membrane transporters that regulate pH homeostasis, cell volume, and transepithelial Na^+ absorption (1-4). At present, the NHE family includes eight isoforms (NHE1-NHE8) that differ in their tissue and subcellular localizations. An intriguing feature of exchangers is that their activities are controlled by various extrinsic factors, including hormones, growth factors, pharmacological agents, and mechanical stimuli (1-4). The regulatory mechanisms of NHE1 and NHE3 isoforms have been studied extensively. For example, NHE1 has been reported to occur via the involvement of a variety of signaling molecules, i.e., calcinuerin B–homologous protein (CHP) (5,6), Ca^{2+}/calmodulin (7,8), 14-3-3 protein (9), Nck-interacting kinase (NIK) (10), and phosphatidylinositol 4,5-bisphosphate (11). Although the interrelationships among these molecules are not known, several transmit signals to the more general NHE1 regulator, the intracellular proton, because many extracellular stimuli control NHE1 activity by changing the apparent affinity for intracellular H^+, probably at the level of the allosteric "H^+-modifier site" (12, and see references 1-4 for review). Therefore, the cytosolic proton is itself an important regulatory factor. Furthermore, we recently have found that CHP is an essential cofactor for supporting the physiological activity of plasma membrane exchangers (6). In this chapter, we focus on two important cytosolic factors, the proton and

CHP. Our recent results show that these two factors are indispensable for the normal function of these exchangers under physiological conditions. In addition, we focus on the role of CHP2 (13), another isoform of CHP.

2. THE PROTON

2.1 The H^+-Modifier Site

In cells, intracellular pH (pH_i) is usually set at a much lower value (~7.2) than that expected from the thermodynamic equilibrium of Na^+ and H^+ (greater than pH 8.0; Figure 1). This "set point" behavior has been attributed to the existence of an allosteric regulatory site(s), often called the "H^+-modifier" site or "pH-sensor." Twenty years ago, Aronson et al. (12) presented evidence for the existence of such a site, based on elegant experiments with renal brush border membrane vesicles. Their data led to the idea that an exchanger becomes active only when a proton occupies the cytosolic H^+-modifier site. Intracellular H^+ has subsequently been shown to activate Na^+/H^+ exchange in several experimental systems (see refs. 1-4 for reviews), including various types of cells expressing endogenous or exogenous exchangers. The H^+-modifier site appears to play several important physiological roles in the regulation of pH_i by the Na^+/H^+ exchanger (Figure 1). First, dissociation of H^+ from the modifier site completely prevents exchange activity and thereby protects cells from alkalosis. Second, H^+ occupation of the modifier site markedly stimulates

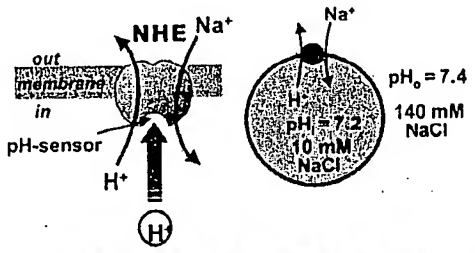

Figure 1. Physiological role of the pH-sensor. The H^+-modifier site (pH-sensor) is different from the H^+-transport site. The pH-sensor plays a critical role in regulation of pH_i.

exchange activity and thereby permits rapid recovery of cells from acidosis. Third, many extrinsic stimuli are capable of delicately modulating the exchange activity in the neutral pH_i range by changing the affinity of H^+ for the modifier site.

Many studies with native or NHE-transfected cells have revealed that, in contrast to the simple kinetics of external Na^+ and H^+, exchange activity obeys complex cooperative kinetics of internal H^+ that involve at least two H^+ binding sites. However, because only the forward mode of exchange was measured in these studies, whether such a regulatory site exists in the various NHE isoforms is not certain. In addition, it was difficult in these studies to distinguish the putative H^+-modifier and H^+-transport sites. Recently, we have tried to dissect kinetically these two H^+-binding sites by measuring EIPA-sensitive $^{22}Na^+$ efflux from cells, i.e., the reverse mode of exchange. We loaded $^{22}Na^+$ into NHE1 transfectants and at the same time clamped pH_i at various values by means of the K^+/nigericin technique. After removing the radioactive preincubation solution, we added a Na^+-free non-radioactive solution (pH 7.4) to start $^{22}Na^+$ efflux from the cells. We observed that intracellular acidification dramatically stimulates $^{22}Na^+$ efflux through NHE1, whereas alkalinization completely inhibits efflux (unpublished observation). If we assume that the Na^+/H^+ exchange obeys a simple counter-transport reaction, the outward-directed H^+ gradient should inhibit $^{22}Na^+$ efflux. The unexpected stimulation of $^{22}Na^+$ efflux by the intracellular proton is consistent with the idea that the exchange activity is regulated by protonation/deprotonation at an H^+-modifier site(s) that is different from the H^+-transport site. The $^{22}Na^+$ efflux of the NHE1 transfectants exhibited a bell-shaped pH_i dependence (unpublished observations, see Figure 2). The activation phase of $^{22}Na^+$ efflux caused by H^+ is very steep in the pH_i range of 7.0 to 7.5, suggesting that two or more protons are involved in exchanger activation. In contrast, strong acidification ($<pH_i$ 6.2) inhibited $^{22}Na^+$ efflux. Presumably, this inhibition results from competition between intracellular H^+ and Na^+ at the transport site. Thus, measurement of $^{22}Na^+$ efflux allowed us to evaluate separately the H^+-modifier and H^+-transport sites, which are related, respectively, to the descending and ascending slopes of the pH_i dependence curve. In fact, the bell-shaped pH_i profile of $^{22}Na^+$ efflux is adequately explained by assuming the binding of multiple protons at the modifier site or sites and a single proton at the transport site. The steep pH_i profile of $^{22}Na^+$ efflux in the neutral pH_i range (see Figure 2) suggests that the modifier site of NHE1 has properties that permit the physiological roles described in Figure 1. However, it should be noted that the complex kinetic behavior of H^+-induced activation has been reported previously: i) transient kinetic studies of membrane vesicles have revealed that the external Na^+

dependence becomes cooperative when the inside of vesicles is acid-loaded (14), suggesting that protonation of the modifier site may change the

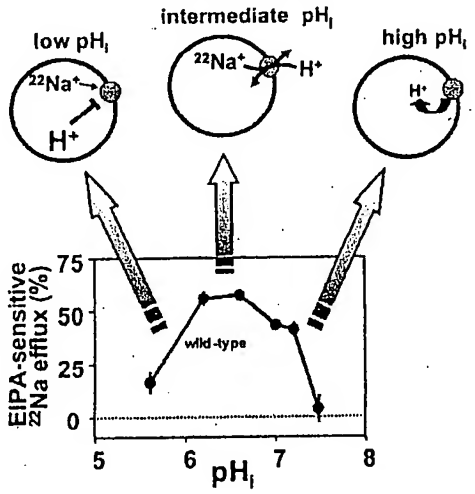

Figure 2. The pH_i profile of $^{22}Na^+$ efflux from NHE1 transfectants. Plotting EIPA-sensitive $^{22}Na^+$ efflux during the initial 3 min indicates that $^{22}Na^+$ efflux is dramatically activated by a slight cytosolic acidification (pH_i 7.0-7.2), reaches a maximum at pH_i 6.6, and then decreases with decreasing pH_i (5.6-6.2). Inhibition by cell alkalinization appears to be due to deprotonation at the modifier site, whereas inhibition by cell acidification appears to be due to competition between H^+ and Na^+ at the transport site.

oligomeric interaction; ii) NHE3, unlike NHE1, is slowly (~5 min) activated by intracellular acidification (15), suggesting that a slow conformational change of NHE3 may be involved; iii) the pH_i and pH_o dependencies of the forward mode of exchange are mutually interrelated (for example, pH_i dependence becomes more acidic with decreasing pH_o [16]), suggesting that the inhibitory interaction of external H^+ with the exchanger may somehow change the properties of the intracellular H^+-modifier site; and iv) intracellular Na^+ is able to activate the exchanger (17), suggesting the possible interaction of Na^+ with the H^+-modifier site. All these data suggest that the H^+-induced activation of the exchangers is regulated by a complex mechanism involving multiple residues and dynamic conformational changes, rather than a simple mechanism such as protonation of ionizable residue(s).

2.2 Critical Regions Involved In pH-Sensing

All NHE molecules can be separated into two large moieties, the amino-terminal transmembrane domain and the carboxyl-terminal hydrophilic domain. On the basis of a cysteine-accessibility analysis, we recently presented a new topology model for NHE1 that consists of 12 membrane-spanning segments with their N- and C-tails in the cytosol (18) (see Figure 3A). Whereas, the N-terminal transmembrane domain exerts the Na^+/H^+ exchange reaction, the cytoplasmic domain functions as the regulatory domain. The initial deletion mutant study (19) provided evidence that the C-terminal cytoplasmic domain plays a critical role in regulating the pH sensing by NHE1. Deletion of the complete cytoplasmic domain resulted in a large acidic shift in the pH_i dependence of the Na^+/H^+ exchange, suggesting that the cytoplasmic domain plays a critical role in pH sensing. Furthermore, the cytoplasmic domain was found to be separated into at least four distinct domains in terms of pH_i sensitivity (20). Deletion of subdomain I (aa 503-595) results in a reduction of pH_i sensitivity, whereas deletion of subdomain III (aa 635-656) results in an enhancement of pH_i sensitivity (Figure 3A). Thus, the former subdomain plays a role in maintaining pH_i sensitivity, as well as in exerting various regulatory functions, such as growth factor–induced activation and ATP depletion–induced inhibition (20). On the other hand, the latter subdomain functions as an autoinhibitory domain and may be involved in Ca^{2+}-induced activation as a Ca^{2+}/calmodulin-binding domain (7,8).

In contrast to the C-terminal cytoplasmic domain, the important region within the N-terminal transmembrane domain that is involved in pH_i sensitivity has not been identified so far. Histidine residues within the transmembrane domain have generally been thought to be good candidates for amino acids involved in the regulation of pH_i sensing, because the imidazole moiety has a pK of 6.2 and is the only amino acid side chain that ionizes within the physiological pH range. Indeed, His^{225} and His^{367} have been identified as important residues for pH sensing by the Na^+/H^+ antiporters of *Escherichia coli* (NhaA) (21) and *Schizosaccharomyces pombe* (Sod2) (22), respectively. However, such residues have not yet been identified in mammalian exchangers. Despite an extensive search for histidine residues involved in pH_i sensitivity, we have failed to identify critical residues within the N-terminal transmembrane domain (S. Wakabayashi, unpublished observation). In addition, His^{35}, His^{120}, and His^{349} within transmembrane segments (TMs) (23) and the cytosolic histidine cluster (HYGHHH) (22) of NHE1 do not appear to be directly involved in the exchange activity. Thus, histidine residues do not appear to

be significantly involved in pH sensing, although we cannot exclude the possibility of the involvement of multiple histidine residues.

Recently, we have focused on the putative 11th transmembrane segment (TM11) and its surrounding regions and found that substituting the Arg^{440} in intracellular loop 5 (IL5) with various residues (Cys, Asp, Glu, His, and Leu) greatly shifts the pH_i dependence of $^{22}Na^+$ uptake to the acidic side, whereas mutations of Gly^{455} and Gly^{456} within the highly conserved glycine-rich region of TM11 significantly shifts it to the alkaline side (unpublished

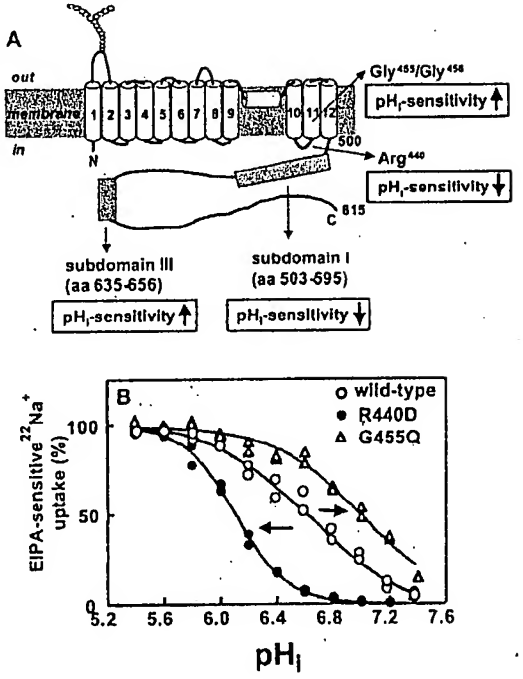

Figure 3. Schematic representation of mutation-sensitive regions involved in pH_i sensing. A, regions or residues for which deletion or point mutations shift the pH_i dependence of exchange toward the acidic or alkaline side. B, pH_i dependencies of EIPA-sensitive $^{22}Na^+$ uptake of cells expressing the mutant NHE1s, R440D or G455Q.

observation, see Figure 3B). Substituting Lys for Arg^{440} did not change pH_i dependence significantly, suggesting that the charge on Arg^{440} is important for pH_i sensing, whereas the alkaline shift that occurred by substituting Gly^{455} with bulky residues such as Gln and Val suggests that a mutation-induced steric hindrance in TM11 may be involved. These two mutant exchangers did not cause changes in the apparent affinities for extracellular

Na^+, H^+, and the inhibitor EIPA, suggesting that the mutations affect a restricted region. Importantly, these mutations shifted the bell-shaped pH_i profile of $^{22}Na^+$ efflux to the acidic (R440D) and the alkaline side (G455Q), respectively. These data suggest that Arg^{440} is an essential residue for regulating pH_i sensing, presumably via a charge-dependent interaction with the cytoplasmic regulatory domain, whereas the Gly residues in TM11 may form a structurally important element for the proper functioning of the putative"pH_i-sensor" of NHE1. Further study is required to clarify how these residues cooperate with the cytoplasmic domain in the regulation of pH sensing.

3. CHP

3.1 CHP1 As An Essential Cofactor

In 1996, Lin and Barber (5) and Kanazawa's group (Osaka U., Japan) have independently identified a novel Ca^{2+}-binding protein, CHP (calcineurin B–homologous protein, here designated CHP1), that interacts with NHE1. CHP1 may be the same protein as the 24-kD NHE1-binding protein identified by another group (24). CHP1 is ubiquitously expressed and is homologous to the calcineurin B subunit (5). CHP1 has four Ca^{2+}-binding motifs (EF-hand), of which two ancestral motifs do not bind Ca^{2+}. The N-terminus of CHP1 is myristoylated. The same protein has been identified independently as a factor (known as p22) required for the vesicular transport of proteins (25). CHP1 has been reported to inhibit calcineurin phosphatase activity (26) and to associate with microtubules (27). CHP1 has been reported to interact with a serine/threonine protein kinase (DRAK2) involved in apoptotic cell death (28) and with a kinesin family member, KIF1Bβ2 (29).

An initial study (5) suggested that CHP1 binds to the cytoplasmic region (aa 566-635) of NHE1. However, on the basis of the *in vitro* pull-down assay, far-Western staining, co-immunoprecipitation, and the observation of co-localization of GFP-tagged CHP1 with NHE1-3, we have shown that CHP1 binds to the juxtamembrane domain (aa 510-530 in the case of NHE1) within the cytoplasmic domain of NHE1-4 (6). The CHP1-binding domain of NHE1 is predicted to form a conserved α-helix similar to that of the calcineurin B–binding domain within the calcineurin A subunit (30). Based on the data for interaction of CHP1 with NHE1 mutant proteins, we have concluded that hydrophobic residues within the CHP1-binding domain are important for interaction, as in the calcineurin A/B complex. Myristoylation and Ca^{2+} binding are not essential for interacting with NHE1. Figure 4

shows fluorescent images of cells expressing the CHP1 fusion protein conjugated with green fluorescent protein (GFP). CHP1 was distributed uniformly in the cytosol of exchanger-deficient PS120 cells (Figure 4). This fusion protein became partly localized in the plasma membrane when exogenous NHE1 was co-expressed (Figure 4B). Thus, NHE1 seems to be the principal target for CHP1 in the membrane.

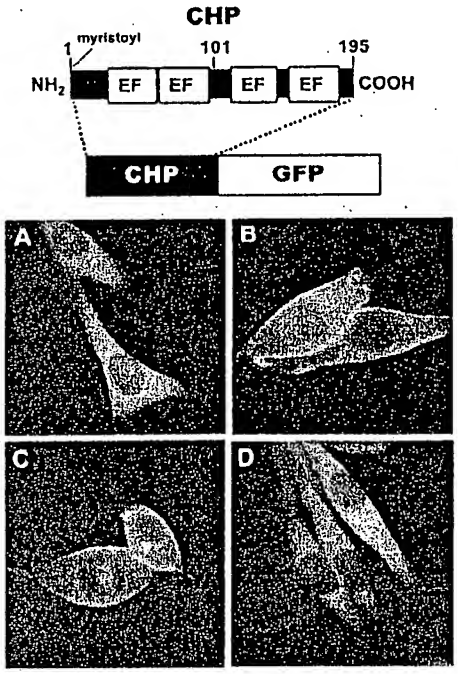

Figure 4. Structure of CHP and subcellular localization of GFP-tagged CHP. CHP1 (B) and CHP2 (C) become localized in the plasma membrane when NHE1 is expressed. A, CHP1-GFP proteins are expressed in exchanger-deficient PS120 cells. D, CHP2-GFP was expressed in cells expressing CHP-binding deficient NHE1 (4R).

Functional analysis revealed the important role of CHP1 in NHE: i) CHP1-binding–deficient mutations of NHE1-3 dramatically reduced exchange activity (V_{max}); and ii) CHP1 depletion by injecting the competitive CHP-binding regions of NHE1 into *Xenopus* oocytes resulted in a dramatic reduction in activity. Thus, CHP1 serves as a common essential cofactor that supports the physiological activity of plasma membrane NHEs.

A single polypeptide for each NHE isoform has generally been considered sufficient for normal exchange activity; however, our data

suggest that multiple exchangers require physical interaction with a common protein, CHP, for expression of the activity.

3.2 Role Of CHP2

Another human CHP isoform (here designated CHP2) has been identified in a human cancer patient (31) (NCBI nucleotide accession number NM022097 with the designation of hepatocellular carcinoma antigen gene 520). CHP2 protein shares a 61% amino acid identity with CHP1. CHP2, like CHP1, contains an N-terminal myristoylation site (Gly2) as well as four EF-hand Ca^{2+}-binding motifs. In contrast to CHP1, however, the expression of CHP2 is extremely low in most human tissues. However, CHP2 was expressed at relatively high levels in malignantly transformed cells, such as hepatoma, colon adenocarcinoma, and leukemia cells, suggesting that CHP2 may be involved in the phenotypic change of NHE properties. Recently, CHP2 was reported to be expressed in the small and large intestine of the rat (32).

We found that i) GFP-tagged CHP2 co-localized with NHEs 1-3, but not with their mutants lacking CHP1-binding ability (see Figure 4); ii) recombinant CHP2 was bound to a MBP fusion protein containing the NHE1 cytoplasmic domain, but not to the fusion protein containing the NHE6 cytoplasmic domain; iii) CHP2 enhanced the exchange activities of NHE1 and NHE3 when co-expressed with them in oocytes; and iv) exchange activity (V_{max}) in cells co-expressing CHP2/NHE1 was comparable with that in cells co-expressing CHP1/NHE1. Therefore, CHP2 competes with CHP1 for binding at the same juxtamembrane domain within the cytoplasmic domain of plasma membrane NHEs and, like CHP1, has the ability to up-regulate the exchange activity. The competition experiment revealed that CHP2 interacts more strongly (5-fold) with NHE1 than does CHP1.

CHP1/NHE1 and CHP2/NHE1 cells responded differently to serum depletion, although they did exhibit a similarly high exchange activity when maintained in serum. The cells co-expressing CHP2/NHE1 but not CHP1/NHE1 exhibited high steady-state levels of pH_i, even in the absence of serum. In addition, exchange activity in CHP2/NHE1 cells was permanently activated in a serum-independent manner. Furthermore, CHP2/NHE1 cells were much more resistant to serum than were CHP1/NHE1 cells (see Figure 5). Surprisingly, 60% of cells expressing CHP2/NHE1 were still viable 10 days after serum starvation, when all CHP1/NHE1 cells had lost viability. The high viability of CHP2/NHE1 cells appears to be due to high pH_i caused by serum-independent activation of NHE1, because cells overexpressing CHP2 were sensitive to serum

starvation when active NHE1 was not expressed or when EIPA was present in the medium.

It is well known that activation of NHE1 is associated with oncogenic transformation (33-36). For example, cells transformed by ras (34, 35) or E7 oncogenes (36) have been shown to maintain a high pH_i in the absence of serum with an accompanying high activity of NHE1, and this response may be one of key factors involved in abnormal cell growth, high resistance to serum deprivation, and abnormal cell invasion. In addition, a high pH_i due to the activation of NHE1 has been observed in various malignantly transformed cells, such as human leukemic (37), malignant glioma (38) and breast cancer cells (39). All these studies suggest that NHE1 becomes permanently activated in many malignant cells; thus, CHP2/NHE1 cells have a property similar to that found in malignantly transformed cells.

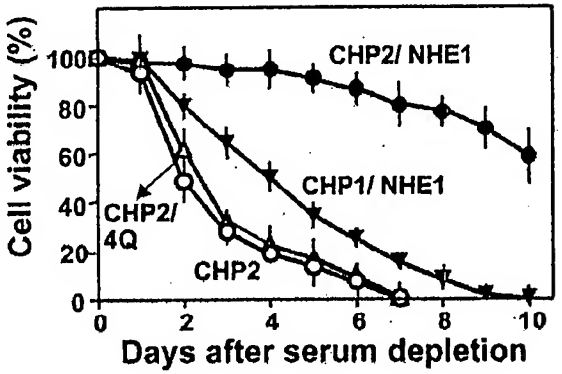

Figure 5. Cells were plated on dishes and serum-depleted on the next day, and the numbers of viable cells remaining were counted on the indicated days. Note the high viability of cells co-expressing CHP2/NHE1, compared with cells co-expressing CHP1/NHE1. Cells expressing only CHP2 or co-expressing CHP2 and CHP-binding-defective mutant 4Q are very sensitive to serum-deprivation.

Figure 6 shows our present working hypothesis. In normal cells, CHP1 interacts with NHE1 at its juxtamembrane region within the cytoplasmic domain. When cells are malignantly transformed, CHP2 becomes expressed at a relatively high level. Because NHE1 interacts with CHP2 more strongly than with CHP1, CHP2 binds predominantly to NHE1 in malignant cells. This preferential binding leads in turn to a serum-independent activation of NHE1. Consequently, activation of NHE1 causes a permanent elevation of

pH_i and thereby results in the abnormal phenotypes of malignant cells, such as high resistance to serum starvation and enhanced cell invasion.

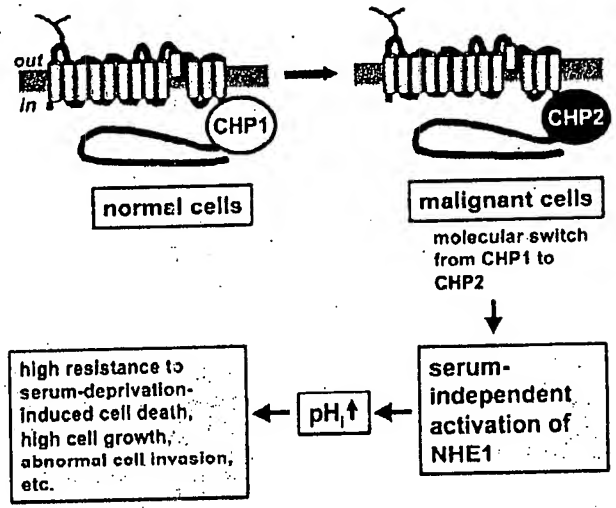

Figure 6. Hypothesis for the role of CHP2 in malignant cells.

4. CONCLUSION

The Na^+/H^+ exchangers are known to interact with various cytosolic factors. In this chapter, we have focused in particular on two important factors, the proton and CHP. We have shown that NHEs basically do not function under physiological conditions unless both factors interact with NHEs. Such observations suggest that these factors are different from other interacting proteins that may be involved in the delicate regulation of exchangers. In this chapter, we also have described the apparent role of CHP2 in maintaining abnormal pH_i in malignantly transformed cells. Future studies, including site-directed mutagenesis, determination of the crystal structure, and physiological experiments using animal models, will be necessary to elucidate more precisely the functions of CHP1 and CHP2 in NHEs and the relationship between these proteins and the "pH-sensor."

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Chapter 4

REGULATION OF EXPRESSION OF THE Na^+/H^+ EXCHANGER IN THE MYOCARDIUM AND OTHER TISSUES

Larry Fliegel
Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

1. INTRODUCTION

1.1 The Importance Of pH Regulation And Na^+/H^+ Exchangers

Eukaryotic cells generate acid and its equivalents through a variety of metabolic processes. Without appropriate regulation by cells, this would result in a steady decrease in intracellular pH (pH_i) (1). For most living cells the maintenance of pH_i in the physiological range is important for a variety of cellular processes including cell growth, differentiation and to maintain an optimum pH for a number of intracellular processes including protein synthesis and the activity of several metabolic enzymes (1-3). Various pH regulatory proteins exist on the plasma membrane to remove excess acid or its equivalent. Perhaps the most important is the Na^+/H^+ exchanger (NHE). The Na^+/H^+ exchanger is a plasma membrane protein that regulates intracellular pH by extruding one H^+ in exchange for one Na^+. Though the contribution of various pH regulatory proteins undoubtedly varies between cell type and species, in the isolated perfused ferret heart the Na^+/H^+ exchanger is estimated to provide approximately 50% of acid extrusion at pH 6.9 (4). At more acidic pH values the Na^+/H^+ exchanger contributes more to pH regulation (5). The proton efflux through NHE1 is estimated to account for 70-80% of the pHi recovery from a carbon dioxide induced acid

load in sheep cardiac Purkinje fibers (6). Bicarbonate based pH regulatory proteins generally contribute less than 50% of total acid efflux in the myocardium. In addition their activity is such that they may be more active at more alkaline pH values (6-9).

NHE1 was the first isoform cloned (10); subsequently NHE2-NHE8 have been discovered. NHE1 is widespread in its cellular distribution and resides in the plasma membrane while NHE2-NHE5 have restricted tissue distributions (11-17). NHE1 is more sensitive to amiloride, its derivatives and the Hoe compounds than isoforms NHE2 and NHE3 (18). Most isoforms are principally in the plasma membrane except NHE3 which has a significant intracellular component (19) and NHE6 and NHE7 are both localized intracellularly (13, 20). NHE8 is newly discovered and is not yet well described. Homology between the isoforms is very high and there are also only small differences between the same isoforms in different species (18, 21). Each isoform represents the product of a different gene (22-24). Hydropathy analysis of the various amino acid sequences indicates that the exchangers have similar predicted membrane topologies, with an N-terminal membrane domain and a C-terminal cytoplasmic domain. For NHE1 it has been shown that there are twelve transmembrane helices and a large C-terminal cytoplasmic domain (25). Figure 1 illustrates a model of the physiological function and overall structure of the NHE1 isoform of the Na^+/H^+ exchanger. In this review we concentrate on the NHE1 isoform, which is the most widespread, the best characterized, and the only plasma membrane isoform present in significant amounts in the myocardium (see

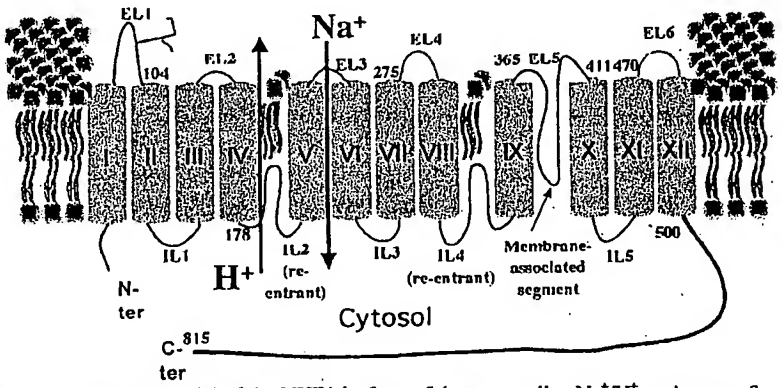

Figure 1. Schematic model of the NHE1 isoform of the mammalian Na^+/H^+ exchanger after Wakabayashi et al (25). IL, intracellular loop, EL, extracellular loop. Positions of re-entrant intracellular loops and a membrane-associated segment are illustrated. Numbering indicates the approximate position of the amino acids within the model. The relative position of phospholipids and their head groups is indicated, as is the direction of Na^+/H^+ exchange.

below). Special consideration is given to the regulation of expression of the Na^+/H^+ exchanger in the myocardium because of its importance in cardiovascular disease.

1.2 Other Physiological Roles Of Na^+/H^+ Exchangers

Na^+/H^+ exchange activity is important in many physiological processes aside from pH regulation. The Na^+/H^+ exchanger assists in regulating sodium fluxes and cell volume after osmotic shrinkage (26, 27). Hyperosmotic solutions rapidly activate the Na^+/H^+ exchanger resulting in sodium entry and cytoplasmic alkalinization. This effect on the Na^+/H^+ exchanger comprises part of a regulatory increase in cell volume, which compensates for shrinkage caused by high external osmolarity (28). The Na^+/H^+ exchanger also initiates increases in intracellular pH that stimulate changes in the growth of cells (26, 29, 30). Cellular alkalinization resulting from Na^+/H^+ exchanger activation is similarly important in oncogenic transformation and is necessary for both the development and maintenance of some transformed phenotypes (31).

Increased activity and increased levels of expression of the NHE1 isoform of the Na^+/H^+ exchanger are important in cell differentiation. A variety of treatments cause cellular differentiation of some cell types and increase mRNA levels of NHE1. This occurs in differentiation of human leukemic cells (HL-60), (32, 33) and during differentiation of P19 (embryonal carcinoma) cells (34). Increased Na^+/H^+ exchanger activity during differentiation is important for enabling the process to occur in some, but not all, cell types (35-37). The NHE1 isoform also acts as a structural anchor for elements of the cytoskeleton (38). It acts as an anchor for actin filaments controlling cortical cytoplasm integrity. The anchoring occurs via links to the actin binding proteins ezrin, radixin, and moesin (39). If this function of the Na^+/H^+ exchanger is disrupted, some cells have impaired organization of focal adhesions, impaired actin stress fiber formation and have an irregular cell shape (39).

The NHE1 isoform of the Na^+/H^+ exchanger might also play a physiological role in moderating apoptosis in some cell types. In pro-β-cell lines, withdrawal of cytokines activates the Na^+/H^+ exchanger elevating intracellular pH (40). This triggers apoptosis via the increased pH_i allowing the proapoptotic protein Bax to translocate to the mitochondria (41). Studies in human leukemic cells suggest that the Na^+/H^+ exchanger may also play a role in apoptosis (42).

1.3 The Na^+/H^+ Exchanger And The Myocardium

Several investigators have demonstrated that the NHE1 isoform of the antiporter is the major isoform present in the myocardium (43, 44). Northern blot analysis of whole heart mRNA first demonstrated the presence of the NHE1 isoform in the myocardium (43). Cloning of the cDNA from the human mammalian myocardium demonstrated that the NHE1 isoform of the protein is identical in sequence to that present in other tissues (44). Northern blot analysis also showed that the NHE1 isoform is present in isolated cardiomyocytes, suggesting that the message shown in whole tissue Northern blots is not from other contaminating cell types (44). Studies have demonstrated that the NHE2-NHE5 isoforms of the antiporter are not present in the myocardium (16, 17, 45-49) or in the case of NHE2 only present in very small amounts (17). NHE6 is present in the myocardium however it has an intracellular localization (12, 13). Overall results have shown that NHE1 is the predominant plasma membrane isoform present in the myocardium and the other isoforms do not play a significant role in plasma membrane proton efflux.

The primary physiological role of the Na^+/H^+ exchanger in the myocardium is quite clear. The protein removes intracellular protons in exchange for extracellular sodium with a 1:1 stoichiometry. In the myocardium however, the Na^+/H^+ exchanger has a steeper relationship between activity and pH than in other tissues. At pH's below 6.5 the protein is maximally activated with a Hill coefficient of activation near 3 (50). This results in maximal activation over a narrow pH range and allows the protein to be most functional during acid load such as during ischaemia.

1.4 The Na^+/H^+ Exchanger In Myocardial Diseases

The Na^+/H^+ exchanger plays a very important role in mediating the damage that occurs to the human myocardium during ischaemia and reperfusion. During cardiac ischaemia protons accumulate resulting in a decrease in intracellular pH. The Na^+/H^+ exchanger then removes intracellular protons and exchanges them for extracellular Na^+ during ischaemia and the subsequent reperfusion. The accumulation of Na^+ may either reduce Ca^{2+} extrusion by the Na^+/Ca^{2+} exchanger or even reverses activity of the protein resulting in Ca^{2+} accumulation (51-53). Excess Ca^{2+} within cells results in cell death, contracture and cardiac arrhythmias. Inhibitors of the Na^+/H^+ exchanger block this damage to the heart. Clinical studies are progressing towards the use of inhibitors of the Na^+/H^+ exchanger in cardiovascular disease (54).

The Na^+/H^+ exchanger is important in growth of some cell types. In relation to this, in the myocardium the Na^+/H^+ exchanger has been implicated in myocardial hypertrophy. Studies by Karmazyn and coworkers (55, 56) have shown that inhibition of the Na^+/H^+ exchanger activity can prevent myocardial hypertrophy in some experimental models. Future studies may explore whether this observation is applicable clinically. Further discussion concerning the role of NHE1 in heart disease is found in Chapters 13-20 of this volume.

2. Na^+/H^+ EXCHANGER EXPRESSION VARIES IN RESPONSE TO THE ENVIRONMENT

The NHE1 isoform of the Na^+/H^+ exchanger is sometimes referred to as the "housekeeping" isoform. It is widespread and is present in virtually all mammalian cells. Traditionally, housekeeping proteins are thought of as unchanging in their levels of expression and are often regarded as "boring' in this matter. However, in the case of the NHE1 isoform of the Na^+/H^+ exchanger, this is far from the truth. This isoform varies greatly in its level of expression in response to environmental stimuli. In some cases the changes are quite dramatic and occur in response to significant physiological events. For example, during differentiation of human leukemic cells (HL-60), there is an 18-fold increase in NHE1 transcription and a 7-fold increase in protein levels (32, 57). We have also shown that NHE1 mRNA levels increase when L6 cells differentiate from myoblast to myotubes (58). NHE1 activity rises during retinoic acid induced differentiation of P19 cells, and blockage of the activity of the Na^+/H^+ exchanger prevents differentiation in this cell type (3). These data support the suggestion that Na^+/H^+ exchanger plays an important role in cellular differentiation. In the kidney and in several renal cell lines, the level of the Na^+/H^+ exchanger increases in response to external acidosis, and this is likely an adaptive mechanism that assists cells in coping with the increased acid load (59-63). This effect may be dependent on glucocorticoids (60) and protein-kinase C in some cell types (63, 64).

In the myocardium, the level of the Na^+/H^+ exchanger varies in response to development, acidosis, ischaemia, hormones and in heart failure and hypertrophy. Our laboratory initially demonstrated that chronic acid treatment of isolated myocytes increases activity of the protein in isolated cardiomyocytes. In addition we demonstrated that treatment of isolated perfused hearts with ischaemia elevates NHE1 mRNA levels (65). Similar observations in the isolated perfused heart showed that ischaemia can elevate NHE1 levels up to seven fold over basal levels (66). Related to these

observations is a study which showed that in humans, NHE1 activity is elevated in end stage heart failure (67). In addition, very recently, Sandmann et al. (68) demonstrated that coronary artery ligation that induces myocardial infarcts in rats, causes significant elevation of NHE1 mRNA levels. Clearly then, ischaemia and acidosis elevate NHE1 in the myocardium. This elevation of the levels of the protein could play an important role in the damage that the Na^+/H^+ exchanger causes during ischaemia and reperfusion of the myocardium.

Several other physiological phenomena have been shown to influence the levels of NHE1 message or protein. Thyroid hormone has been shown to strongly influence the levels of NHE1 message and protein in the myocardium. An early study showed that hyperthyroidism increases mRNA levels of the Na^+/H^+ exchanger (69). Recently, we (70) have shown that treatment of cardiac myocytes with 3,5',3'-triiodothyronine results in an increased expression of Na^+/H^+ exchanger protein. Also, compared with euthyroid animals, hypothyroid rats expressed decreased amounts of the Na^+/H^+ exchanger protein.

Developmental regulation of the Na^+/H^+ exchanger has been well established. An early report confirmed that there is greater sarcolemmal Na^+/H^+ exchanger activity in rat, newborn myocytes in comparison to the adult (71). In rabbits increased levels of NHE1 mRNA correlate with the changes in Na^+/H^+ exchanger activity suggesting that increased mRNA expression may account for the increased activity of the protein (72). We recently examined Na^+/H^+ exchanger protein levels during postnatal development in mice. Protein levels increased after embryonic day 18 and peaked at 14 days of age in the heart, lung, liver, kidney, and brain. The greatest rise in NHE1 protein expression occurred in the heart with over 6-fold increases in protein expression when comparing 2-week-old hearts to neonatal hearts. The smallest increase was in the brain where there were only 30% increases when comparing 2 week old hearts to neonatal hearts (73). To examine transcriptional regulation of expression of the Na^+/H^+ exchanger in fetal mice we made transgenic mice with the NHE1 promoter driving expression of green fluorescent protein. The level of NHE1 transcription varied between tissues and with the stage of development. The highest expression was in the heart and liver of 12- to 15-day-old mice. This declined with age so that the transcription in these tissues was reduced in the 15-day-old mice compared to the 12-day-old mice; and in 1-day-old neonates expression of the reporter was not detectable above background levels (73).

The level of the NHE1 protein is also elevated in some models of muscle hypertrophy. NHE1 message levels were increased markedly in a model of cardiac hypertrophy, pressure-overload following aortic constriction. In addition stretch of myocytes on deformable plates could elevate NHE1 message levels (74). NHE1 message levels are elevated 2-fold in right ventricles of rat hearts in a model of monocrotaline induced hypertrophy (75). In another model, the spontaneously hypertensive rat, it has also been shown that activity of the Na^+/H^+ exchanger is increased (76). Because of the role that the Na^+/H^+ exchanger plays in hypertrophy, elevated expression of the NHE1 protein could have important physiological effects in the etiology of the disease.

3. TRANSCRIPTIONAL REGULATION OF THE Na^+/H^+ EXCHANGER GENE

3.1 The Human, Rabbit And Porcine NHE1 Promoters

The first Na^+/H^+ exchanger promoter cloned was the human NHE1 isoform. The NHE1 promoter has been cloned from several species though the mouse NHE1 promoter (considered below) has been analyzed in the greatest detail and under more varied physiological conditions. The human Na^+/H^+ exchanger genomic clone was isolated in 1991 (77). The clone contained 1377 bp upstream of the start site of transcription. This early study did not directly test the elements of the gene involved in transcriptional regulation but analysis of the sequence showed that the gene contained a cyclic AMP response element, two CAT boxes, three AP-1 sites, and three GC boxes (77). Other studies have shown that the human NHE1 gene is located on chromosome 1 (78). Analysis of its structure shows that the gene contains 12 exons and 11 introns. The first exon is 1132 bp in length and is separated from the second exon by a 41.5 kb intron. The other introns vary in length from 4.2 to 0.37 kb (77).

Horie et al. (64) examined the role of the transcription factor AP-1 in regulation of NHE1 expression in cultured renal proximal tubule cells. Acid incubation increased AP-1 activity and the activity of a reporter consisting of 6 tandem AP-1 binding sites. However a direct effect of acid on the NHE1 promoter was not shown. Kolyada et al. (79) used footprint analysis of the human NHE1 gene to demonstrate that 4 principal regions of the promoter bound nuclear proteins (from most proximal to distal these were designated A to D). Deletion of the promoter up to the D region did not reduce promoter activity in reporter constructs examined in HepG2 (hepatocyte) cells, NIH 3T3 cells (fibroblasts) or in vascular smooth muscle A7r5 cells. In HepG2 and smooth muscle cells, deletion up to the D region caused stimulation of activity of the promoter, however in fibroblasts the opposite effect was seen with a minor reduction in promoter activity. Deletion or

substitution of nucleotides within D region caused decreased activity of the promoter (79). By using bandshift analysis it was suggested that the C/EBP family of transcription factors may be responsible for this regulation (79). Kolyada et al. (80) also demonstrated that disrupting the D region suppressed transcription in vascular smooth muscle and hepatic cells.

The rabbit NHE1 promoter is homologous to the human gene, especially in the more proximal regions of the promoter. Analysis of the activity of the rabbit promoter demonstrated that a 4.7 kb fragment of the promoter was only 40% as active as a 1.1-kb fragment of the promoter. This suggested that negative regulatory elements exist upstream of the 1.1 kb region (81). The porcine NHE1 promoter is also homologous to the human, rabbit and mouse promoters in the proximal 500 bp of the 5' flanking region. Several consensus elements for the transcription factors AP-1, C/EBP and Sp1 were conserved between pig and human while only AP3 and PEA3 were found in the porcine promoter (82).

3.2 Regulation Of The Mouse NHE1 Promoter.

3.2.1 Proximal Regions Of The Mouse NHE1 Promoter

The mouse NHE1 promoter was cloned in our laboratory in 1995 (83). Analysis of the sequence showed that the location of the splice site of the first intron was identical in the human and mouse genes (84). Of the putative regulatory elements found in the sequence, we demonstrated that the transcription factor AP-2 is important in regulating basal expression in several cell types. The AP-2 binding site is located at bp -95 to -111. Deletion of regions of the promoter upstream of the AP-2 site reduced the basal activity of the promoter about 70% in fibroblasts (83) and about 40% in P19 embryonal carcinoma cells (34). The binding of this transcription factor to the gene was confirmed by gel mobility shift analysis and DNase I footprinting analysis using nuclear extracts from NIH 3T3 cells. We confirmed that in intact cells this transcription factor is important in basal expression of the promoter (83). However more recently (85) we examined transcriptional regulation of the promoter in transgenic mice with a knockout of the AP-2α transcription factor. In 18-day old embryos from AP-2α null mice, there was a large increase in Na^+/H^+ exchanger protein expression in the brain (85). It may be that another member of the AP-2 transcription factor family may be responsible for activation of the NHE1 gene. To date, three proteins have been placed in this family: AP-2α, AP- 2β, and AP-2γ. Although they recognize a common DNA binding sequence, members differ somewhat in their expression patterns (86). Future studies will have to

determine which isoform of the AP-2 transcription factor is important in basal NHE1 transcription.

It is also interesting that the NHE1 promoter activity is upregulated in cellular differentiation. P19 cells are a model of embryonic determination and differentiation and retinoic acid treatment induces the development of neurons, astroglia, and microglia cells. We have shown that with retinoic acid induced differentiation there is an early and rapid 10-fold increase in NHE1 transcription which involves the proximal AP-2 site (34). This promoter activity declines after differentiation ceases. In addition, the promoter is activated in differentiation of L6 muscle cells though this appears to involve a more distal region of the gene (58).

A highly conserved poly (dA:dT) region of the NHE1 promoter is located at bp -155 to -169 of the mouse promoter. Deletion or mutation of this region results in dramatic decreases in basal promoter activity of both L6 and NIH 3T3 cells. This region is protected by nuclear extracts of L6 and NIH 3T3 cells and can act as an enhancer in a foreign promoter (87). The high mobility group family of nuclear proteins bind to this region of the promoter (88). These proteins are preferentially expressed in rapidly dividing, malignant and undifferentiated cells and may be important in growth and differentiation (89) suggesting this may be one mechanism of regulating the Na^+/H^+ exchanger in cellular growth and differentiation.

3.2.2 Distal Regions Of The Mouse NHE1 Promoter

More recently, some more distal regions of the mouse NHE1 promoter have been shown to be involved in regulation of the gene in response to various physiological stimuli. We showed (90) that a 1.1 kb fragment of the NHE1 promoter increases in transcriptional activity in response to a variety of mitogenic stimuli. The stimuli include serum, insulin, thrombin and epidermal growth factor. Phorbol esters increased transcriptional activity while a serine/threonine protein kinase inhibitors including [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine] inhibited the effect. Surprisingly the protein kinase inhibitors PD-98059, genistein and GF-109203X stimulated activity of the promoter. A region of the gene from –800 to –1085 was implicated in this regulation.

Further analysis (91) has suggested that one important type of transcription factor involved in the distal region of the gene is the chicken ovalbumin upstream promoter transcription factor (COUP-TF) type I and II. The nucleotides at –841 to –800 bp upstream of the start site bound COUP-TF's. Mutations within this site could block transcription factor binding and could prevent this region from acting as a strong enhancer. Further

implicating this transcription factor in regulation of the gene was the evidence that overexpression of the COUP-TF proteins activated the NHE1 promoter (linked to a reporter) and increased the endogenous NHE1 message in NIH 3T3 cells. The COUP-TF transcription factors were also implicated in the upregulation of NHE1 expression that occurs in retinoic acid induced differentiation of P19 cells. A complicating factor is that the thyroid hormone receptor $Tr\alpha_1$ is also implicated in regulation of this region of the promoter (70). The –841 to –800 element interacts with $Tr\alpha_1$, a nuclear hormone receptor that is involved in mediating the effect of thyroid hormone. COUP-TF and $Tr\alpha_1$ do not appear to heterodimerize however the binding of $Tr\alpha_1$ transcription factor to the element could reduce subsequent COUP-TFI binding indicating that the binding sites do overlap. Transfection of cells with $Tr\alpha_1$ caused increases in activity of several promoters that had tandem copies of the element inserted. These results suggested that the $Tr\alpha_1$ nuclear receptor activates the NHE1 promoter through interaction with this element. We have demonstrated that expression of NHE1 initially increases following birth and then the protein levels decline with time (73). The time course of this expression is similar to that of changes in thyroid hormone suggesting that T3 may be affecting NHE1 levels during development through this element.

3.3 Regulation Of The NHE1 Promoter In The Myocardium

We examined the factors involved in regulation of the NHE1 promoter in the mammalian cardiomyocyte. The 1.1 kb region of the mouse promoter was active in transfected cardiomyocytes. Serum stimulated activity of the promoter in cardiomyocytes, however a surprising result was that acidosis did not directly activate the NHE1 promoter in this assay. We examined the effect of transfection of cardiomyocytes with constructs of the promoter with distal regions removed up to the AP-2 site. We found that there was a 4-fold decrease in NHE1 promoter activity compared with the intact gene. This showed that the distal regions are important in NHE1 expression in the myocardium (92). Mutation of the AP-2 site combined with deletion of upstream regions of the promoter, resulted in almost total removal of promoter activity. DNA mobility shift binding assay confirmed that AP-2 or an AP-2 like protein are involved in regulation of the NHE1 promoter. However it was interesting that for the AP-2 binding region, the mobility shift induced by cell extracts was of different size than that induced by pure AP-2α protein (92). This suggested that either another isoform of AP-2 is involved in regulation in the myocardium, that the AP-2 protein is modified

post-translationally before binding, or that it heterodimerizes with another transcription factor.

Other regions of the NHE1 promoter are involved in regulation of expression and have only been briefly investigated. DNase I footprinting analysis showed that the poly(dA:dT) region is protected by heart nuclear extracts (88) suggesting it may be important in regulation of expression in the myocardium. We (70) have also shown that thyroid hormone affects expression of the NHE1 protein in the intact animal and in isolated cardiomyocytes. Treatment of isolated cardiomyocytes with thyroid hormone increased the binding of proteins of nuclear extracts to the –841 to –800 element. This suggests that this distal element may be important in thyroid hormone regulation of expression in the myocardium. Further studies are necessary to learn what other regions are important in regulation of expression in the myocardium. Figure 2 illustrates a schematic diagram of the mouse NHE1 promoter with several of the regions important in transcription illustrated.

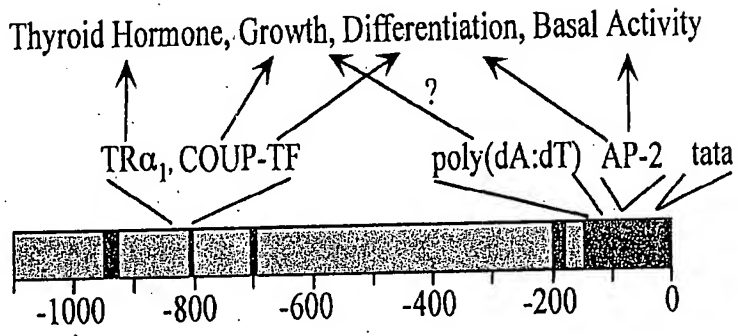

Figure 2. Schematic model of the initial 1100 bp of the mouse NHE1 promoter. The sites of binding of transcription factors known to be important in regulation of activity are indicated. Darker shaded regions indicate a region of homology to the human NHE1 promoter. The site of the TATA box is indicated. Analysis of homology was with the program MacVector™. Arrows indicate the physiological phenomenon associated with the regulatory element.

4. CONCLUSION

Studies have demonstrated that the NHE1 isoform of the Na^+/H^+ exchanger is regulated in response to a variety of environmental stimuli. The NHE1 message and protein are developmentally regulated, are regulated in response to differentiation, in response to acidosis, ischemia and hormones. The changes in expression often correlate with these

physiological events and in some cases blockage of activity of the Na^+/H^+ exchanger has been shown to prevent the physiological phenomenon. The mechanism by which regulation of the Na^+/H^+ exchanger responds to the environment has been partially elucidated. The transcription factor AP-2 is at least partially responsible for basal regulation of the promoter and is implicated in increased expression in differentiation of some cell types. The transcription factor AP-1 may have a role in regulation of expression in response to acidosis. COUP-TF appears to be important in increased expression in response to growth and mitogenic stimuli. Thyroid hormone increases expression of the Na^+/H^+ exchanger in the myocardium and its effects appear to be mediated at least partially through the nuclear hormone receptor $Tr\alpha_1$. A picture is emerging of regulation of expression of the Na^+/H^+ exchanger that is mediated at least partially through environmentally responsive transcription factors. Future studies may further confirm the universality of this observation.

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Chapter 5

Na-H EXCHANGE FUNCTION IN COLONIC EPITHELIAL CELLS

Henry J. Binder, Vazhaikkurichi M. Rajendran, John Geibel
Departments of Internal Medicine, Cellular and Molecular Physiology, and Surgery, Yale University, New Haven, Connecticut, U.S.A.

1. INTRODUCTION

Epithelial cells of the mammalian colon have multiple functions but are primarily responsible for the absorption and secretion of fluid and electrolytes (1). Normally, fluid, Na and Cl are absorbed and K and HCO_3 are secreted by colonic epithelial cells, while multiple factors including hormones and diet may modify absorptive and secretory processes both qualitatively and quantitatively. There are several unique features of colonic epithelial cells with significant heterogeneity in their function. Epithelial cell function both of the proximal and distal segments and of the surface and crypt cells of the colon are not identical, while the mechanism of Na and Cl transport may differ in several species. In contrast to small intestinal epithelial cells that are critical for nutrient absorption, colonic epithelial cells absorb only fluid, electrolytes and short-chain fatty acids (SCFA) and do not participate in the absorption of carbohydrates, amino acids and fat except in the neonatal period.

Central to these epithelial cell functions are several membrane ion transport processes, including Na-H exchange (NHE). To date, multiple NHE isoforms (at least eight) have been cloned and identified (1-6) while in colonic epithelial cells four different NHE (i.e., NHE-1, NHE-2, NHE-3 and Cl-NHE) isoforms are present with localization to different cells and specific cell membranes (4,7,8). Two of these NHE isoforms (e.g, NHE-3, Cl-NHE) are linked to Na absorption; NHE-1 isoform is associated with the regulation of cell function, i.e., intracellular pH (pHi) and cell volume; while the

function of Cl-NHE and NHE-2 isoforms is controversial. Aldosterone, which can produce profound changes in Na absorption in many epithelia including the colon, has specific (and varied) effects on the several NHE isoforms that have been identified in the colon. This Chapter will summarize present information regarding the distribution, function and regulation of NHE isoforms in colonic epithelial cells with emphasis on studies performed in the rat large intestine.

2. APICAL MEMBRANE NHEs

Transport studies in the distal colon of the rat and rabbit have demonstrated substantial differences in the mechanism of Na absorption in these two species (1). In the rabbit distal colon Na absorption is electrogenic, Cl-independent and inhibited by low-dose amiloride, while on a molecular basis Na absorption across the apical membrane occurs via an epithelial sodium channel (ENaC). In contrast, Na absorption in the rat distal colon is electroneutral, Cl-dependent, inhibited by high-dose (but not low-dose) amiloride and represents Na-H exchange. Aldosterone, either via its subcutaneous infusion or secondary to dietary Na depletion, results in the induction of electrogenic Na absorption with a parallel down-regulation of electroneutral Na absorption, i.e., Na-H exchange at the apical membrane.

A series of Na uptake studies with apical membrane vesicles (AMV) has characterized the presence of Na-H exchange in rat distal colon (9-16). The unique observation of these studies is the absence of a proton modifier site that has regularly been identified in studies of NHE activity in intact tissues and cells (10). We suspect that the absence of proton activation of Na uptake is related to the role of apical membrane NHE function in transepithelial Na movement and not in pHi regulation, but cannot exclude the possibility that such a proton modifier site had been altered during vesicle membrane preparation. The kinetic parameters of Na-H exchange in AMV isolated from rat distal colon have been compared to those of the colonic NHE isoforms when expressed in PS120 cells (4) (Table 1). Although the Km for Na for NHE activity in AMV from rat distal colon was similar to that for NHE-2 and NHE-3 isoforms, Ki for amiloride for NHE activity in AMV (27 μM) was similar to that of NHE-3 isoform (39 μM) and not that of either NHE-2 or NHE-1 isoforms (1-3 μM). Although all Na-H exchangers are completely inhibited by 1 mM amiloride, different amiloride analogues can distinguish the several NHE isoforms. For example, S3226 inhibits NHE-3 isoform and not NHE-2 isoform. Table 1 demonstrates that the Ki for S3226 is almost identical for NHE activity in AMV and NHE-3 isoform expressed in PS120 cells indicating that the NHE activity in AMV represents NHE-3 isoform and not NHE-2 isoforms. (15,17).

Table 1: Comparison of NHE Activity in Colonic AMV to Expressed NHE-2 and NHE-3 Isoforms

	Colonic AMV	NHE-2	NHE-3
Km for Na (μm)	10.6	18	17
Ki for amiloride (μm)	27	1-3	39
Ki for EIPA (μm)	0.4	1	8
Ki for S3226 (μm)	0.19	80	0.23

Based on data presented in ref #4, 10, 17, 45, 57

In view of the down-regulation of both electroneutral Na absorption and NHE activity by aldosterone studies were also performed that examined whether aldosterone had differing effects on the activity, protein expression and message abundance of NHE-2 and NHE-3 isoforms. We observed that aldosterone abolished both Na absorption in studies of transepithelial HCO_3-dependent Na movement across intact tissue and total NHE activity in AMV studies (18,19). Similarly, NHE-3 isoform activity, protein expression and mRNA abundance were also almost completely inhibited by aldosterone (15) (Table 2). In contrast, aldosterone reduced NHE-2 isoform activity by 80%, protein expression by 33% and mRNA abundance by 40%. These observations suggest that NHE-3 isoform is more closely responsible for HCO_3-dependent transepithelial Na movement than is NHE-2 isoform. This conclusion is consistent with two prior observations regarding the immunochemical distribution of NHE-3 and NHE-2 isoforms to surface and crypt cells (8,20). NHE activity [i.e., Cl-independent NHE activity (see below)] represents a surface cell and not a crypt cell function and NHE-3 isoform protein expression and mRNA abundance are restricted to surface cells. In contrast, NHE-2 immunoreactivity is present in both surface and crypt cells. Further, in the rabbit distal colon where neither electroneutral Na absorption nor functional NHE activity are present, NHE-2, but not NHE-3 immunoreactivity is present (20).

Similar type studies were performed by Cho et al in rat small and large intestine, but somewhat different results were reported (53). Using a supraphysiologic amount of dexamethasone (600 μg/kg body wt) an increase in NHE-3 isoform mRNA abundance was identified in ileal villous and proximal colonic surface cells (but no change was observed in surface cells of distal colon). In parallel studies of NHE isoform mRNA abundance, these investigators observed that aldosterone enhanced only NHE-3 isoform mRNA abundance in proximal colon (54). The differences between these results and those reported from our laboratory may be related to different

amounts and routes of administration of aldosterone employed in these studies.

Table 2: Effects Of Aldosterone On Nhe Isoform Function

	Proxical Colon	Distal Colon
NHE 1		
Activity	→	→
mRNA	→	→
Protein	ND	ND
NHE 2		
Activity	↑ 700%	↓ 90%
mRNA	↑ 200%	↓ 35%
Protein	↑ 300%	↓ 33%
NHE 3		
Activity	↑ 200%	↓ 100%
mRNA	↑ 300%	↓ 90%
Protein	↑ 700%	↓ 75%
CL-NHE		
Activity	ND	↑ 60%
mRNA	ND	↑ 200%

ND = Not Done; → = unchanged

Data based on results reported in ref # 15, 36.

Recent studies of electroneutral Na-Cl absorption that have been performed in the jejunum of NHE-2 isoform and NHE-3 isoform knockout

mice confirm that NHE-3 isoform, and not NHE-2 isoform is primarily responsible for NHE-mediated Na absorption (21,22). Unexplained is a residual Na absorptive mechanism in the NHE-3 isoform knockout mice that most likely is not NHE-2 isoform. Similar experiments have not as yet been performed in the distal colon.

The conclusion of these several observations is that NHE-3 isoform is closely linked to that component of NHE activity in surface cells that is responsible for HCO_3-dependent transepithelial Na movement, but that although NHE-2 isoform may also be at times associated with transepithelial Na movement, it is likely that NHE-2 isoform has other functions that have not as yet been identified. The role of NHE-2 isoform in SCFA-stimulated Na absorption is discussed later in this chapter.

The majority of studies that have examined the effect of aldosterone on NHE function and Na transport have examined the *induction* of electrogenic Na absorption and ENaC expression and indicate that evidence of ENaC expression can be observed within a few hours of aldosterone administration (18,19). Parallel to the induction of ENaC expression electroneutral Na-Cl absorption (i.e., NHE function) is *inhibited* by aldosterone (18,19). In studies that have evaluated both the inhibition of electroneutral Na-Cl absorption and the reappearance of electroneutral Na-Cl absorption following the removal of aldosterone stimulation, it is apparent that aldosterone regulation of NHE function requires forty-eight to sixty hours consistent with a genomic effect (23). This time period represents the turnover time for crypt cells to migrate along the crypt to become mature surface cells suggesting that the regulation of NHE function by aldosterone most likely occurs in cells located in the base of the crypt, but is not expressed until those cells become functional surface cells.

Although mineralocorticoids induce ENaC, inhibit electroneutral Na-Cl absorption and stimulate active K secretion, glucocorticoids have quite different effects on ion transport in the rat distal colon (19,24,25). Several studies of glucocorticoid action had been performed with methylprednisolone and dexamethasone that indicated effects that were similar to those of aldosterone (26,27). Subsequent experiments established that most of these studies used amounts of steroids that interacted primarily with the mineralocorticoic receptor. Investigations that employed either very low doses of methylprednisolone or RU-28362, a glucorticoid-specific receptor agonist, have provided unequivocal evidence that activation of the glucocorticoid receptor results in *stimulation* of electroneutral Na-Cl absorption without any evidence of an induction of ENaC (19,24,25). This up-regulation of electroneutral Na-Cl absorption presumably represents stimulation of apical membrane NHE function, but experiments to analyze the effect of glucocorticoids on specific apical membrane NHE isoforms (e.g., NHE-2 and/or NHE-3 isoforms) have not been performed. In the rabbit ileum, methylprednisolone increases brush border NHE activity solely

as a result of increasing NHE-3 isoform expression (without any change in NHE-2 isoform expression) (7,52). In contrast, in the avian colon, transepithelial Na absorption is associated to NHE-2 isoform (55). In these latter studies in the large intestine of the Hubbard chicken aldosterone, secondary to dietery Na depletion, increased apical membrane NHE-2 isoform activity and NHE-2 protein expression (55). Whether the difference in the role of NHE-2 and NHE-3 isoforms is solely related to species, intestinal segment studied or other factors requires resolution. In contrast to the stimulation of active K secretion by aldosterone, RU-28362 had no effect on K transport in the rat distal colon (19).

Metabolic acidosis has also been used to assess the regulation of colonic NHE isoforms. Similar type studies have been extensively performed in renal tubules. Lucioni et al (56) observed that metabolic acidosis induced by feeding NH_4Cl increased the activities, mRNA abundance and protein expression of both NHE-2 and NHE-3 isoforms, but similar to aldosterone, did not affect NHE-1 isoform. Aldosterone levels were not reported in these animals with metabolic acidosis.

SCFA are produced by colonic bacteria from non-absorbed carbohydrate, are the primary anion in stool and are absorbed in the mammalian colon. Transport studies of SCFA-dependent Na absorption proposed a model that included 1) an apical membrane NHE together with 2) a mechanism of SCFA uptake across the apical membrane (i.e., SCFA-HCO_3 exchange and/or non-ionic diffusion of SCFA) and 3) SCFA-Cl exchange (28). The observations that SCFA-dependent Na absorption is inhibited by amiloride and aldosterone are consistent with this model (29,30). In contrast, cAMP, which inhibits HCO_3-dependent Na absorption by virtue of its inhibition of NHE activity, did not alter SCFA-dependent Na absorption (30). Subsequent in vitro transport studies that included using both 50 μM HOE694, a concentration of this amiloride analogue that inhibits NHE-2, but not NHE-3 isoforms, and S3226, another amiloride analogue that inhibits NHE-3 isoform, but not NHE-2 isoform, resulted in the following conclusions: 1) cAMP inhibits NHE-3, but not NHE-2 isoform function; 2) HCO_3-dependent Na absorption requires NHE-3, but not NHE-2 isoform; 3) NHE-3 isoform is involved in SCFA-dependent Na absorption; but 4) if NHE-3 isoform is inhibited, SCFA-dependent Na absorption will also be maintained by NHE-2 isoform (17). Thus, these observations explain the failure of cAMP to inhibit SCFA-dependent Na absorption despite its inhibition of NHE function and indicate at least one role for NHE-2 isoform in regulating Na movement across the apical membrane. These observations also provided the basis for an improvement in oral rehydration therapy for acute diarrhea by the addition of an amylase-resistant starch to oral rehydration solution (31). Such a formulation resulted in an increase in SCFA production which in turn led to enhanced colonic Na and fluid absorption with a decrease in stool output.

The effect of aldosterone on electroneutral Na absorption differs strikingly in the rat **proximal** colon from that in the rat distal colon (32,33). In contrast to aldosterone's well-delineated effects on Na absorption in the rat distal colon that are described above and summarized in Table 2, aldosterone **increases** electroneutral Na absorption in the rat proximal colon. Although glucocorticosteroids also increase electroneutral Na absorption in the rat distal colon, the effect of aldosterone in the proximal colon represents a specific mineralocorticoid effect in that: 1) aldosterone and RU-28362, a specific glucocorticosteroid-specific agonist, have differing effects on Na and K transport and 2) spironolactone, a specific mineralocorticoid-receptor antagonist, blocks the effects of aldosterone, but not those of RU-28362 (33). Further, aldosterone increases NHE activity in AMV that represents a 3.6 fold increase in Vmax without any change in Km (15). As a result, aldosterone's effect on NHE activity represents either an increase in turnover rate of an existing NHE or an induction of the synthesis of new NHE proteins.

Studies were also performed to determine whether this increase in NHE activity represented NHE-2 isoform or NHE-3 isoform function or both (15). Similar to studies in rat distal colon, the amiloride analogue, HOE694, was employed to distinguish between NHE-2 and NHE-3 isoform activity. NHE-3 isoform was also the predominant NHE isoform in AMV of rat proximal colon representing 89% of total NHE activity. Aldosterone increased both NHE-2 and NHE-3 isoform activities, but the increase in NHE-2 activity was substantially greater than that of NHE-3 isoform activity such that in AMV from proximal colon of aldosterone rats NHE-3 isoform activity was 69% of total NHE activity. Western blot and northern blot analyses of normal and aldosterone rats revealed that aldosterone increased the protein expression and mRNA abundance of both NHE-2 and NHE-3 isoforms indicating that aldosterone's enhancement of NHE activity represents pretranslational regulation.

2.1 Cl-dependent NHE In Crypt Cells

Long-standing models of surface/crypt cell function in the mammalian colon indicate that absorptive processes are present in surface cells and secretory processes in crypt cells with the additional implicit concept that absorptive processes are **not** present in crypt cells (34). To study crypt cell function directly methods were established to isolate individual crypts and to perform microperfusion studies in which the open (or top) end of the crypt was cannulated and the blind (or bottom) end of the crypt was punctured; this experimental model allows access to both the apical and basolateral membranes of intact crypts (35-37). This technique is based on methods

previously developed for the microperfusion of renal tubules. Furthermore, this method permitted collection of luminal fluid during modifications of the perfusate bathing either the apical or basolateral surfaces. During perfusion with a HCO_3-Ringer solution Na-dependent fluid *absorption* was consistently demonstrated while the addition of cyclic AMP or vasoactive intestinal peptide (VIP) to the bath solution resulted in net fluid *secretion* (35). These crypts are isolated without their surrounding pericryptal sheath that contains myofibroblasts, cells that produce eicosinoids. We, therefore, concluded that the constitutive transport process in the crypt epithelial cell is an absorptive process, but that net fluid secretion is a regulated process representing the stimulation of myofibroblasts in the pericryptal sheath by one or more neurohumoral agonists released from lamina propria cells to activate eicosinoid production which in turn induce active Cl secretion. Thus, in the basal state either in vivo or in vitro with intact mucosa, net fluid secretion is most likely a consequence of Cl secretion secondary to resting cholinergic tone.

Prior studies had established that NHE-3 isoform mRNA and protein were present in surface cell epithelia and **not** in crypt cells (8). Since NHE-3 isoform had been linked to colonic HCO_3-dependent Na absorption, there was no ready mechanism to explain the demonstration of Na-dependent fluid absorption in the microperfused isolated colonic crypt. Therefore, studies of Na uptake by AMV isolated from the crypt epithelial cells were performed and established the **absence** of Cl-independent NHE activity in crypt AMV. In contrast, Cl-dependent NHE (subsequently referred to as Cl-NHE) activity was identified and is the only detectable NHE activity in crypt AMV (13,14,16) (Figure 1). In AMV isolated from surface cells the predominant NHE activity was Cl-independent with 26% of total NHE activity being Cl-dependent (Figure 1) (13,14). Thus, Cl-independent NHE activity is present only in surface cells while Cl-NHE is the *sole* detectable NHE present in apical membranes of crypt cells and is primarily present in crypt (and not in surface) cells. In parallel studies Na-dependent pHi recovery to an acid load in crypt epithelial cells was also determined during microperfusion of intact crypts and provided evidence that Na-dependent pHi recovery to an acid load had an *absolute* requirement for Cl. This observation is consistent with the presence of an apical membrane Cl-NHE in crypt cells.

Subsequent studies revealed that the Cl-dependence of Cl-NHE represented a Cl channel, not a Cl-anion exchange (16). The evidence in support of this conclusion included: 1) 100 μM DIDS, a concentration that inhibits Cl-anion exchange, did not inhibit Cl-NHE activity in crypt AMV; 2) 1 mM DIDS, a concentration that inhibits non-CFTR Cl channels, and NPPB, a non-specific Cl channel blocker, inhibited Cl-NHE activity; 3) NPPB also prevented the Cl-dependency of Na-dependent pHi recovery to an acid load; and 4) a blocking antibody to CFTR and glybenclamide, a known CFTR inhibitor, inhibited 40% of Cl-NHE activity and Cl-

dependency of Na-dependent pHi recovery to an acid load, respectively. These several observations were interpreted to indicate that Cl-NHE represented a NHE isoform, either an existing NHE isoform (s) or a unique isoform that was coupled to one or more Cl channels. From these initial studies, we could not exclude the possibility that Cl-NHE represented an unrelated transport protein with both NHE and Cl channel activities.

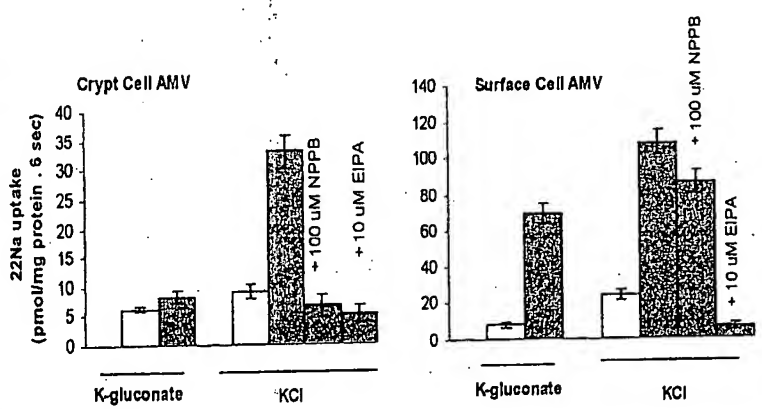

Figure 1: [H] gradient-driven ^{22}Na uptake (NHE activity) in the presence and absence of extravesicular Cl in apical membrane vesicles (AMV) prepared from crypt cells (left panel) and surface cells (right panel). Open bar represents experiments performed in the absence of [H] gradient, while hatched bar represents experiments performed with an outward directed [H] gradient (pHi/pHo:5.5/7.5). In crypt cell AMV, all NHE activity is Cl-dependent; i.e., no Cl-independent NHE activity was identified. In contrast, in surface cell AMV, NHE activity is primarily Cl-independent with 26% of total NHE activity being Cl-dependent. These results were adapted from data presented in ref. #13 and Rajendran, V.M., unpublished observations.

Further studies that included kinetic analyses of Cl-NHE in crypt AMV were also performed: the Ki for amiloride for Cl-NHE was substantially higher (480 μM) than those of other colonic NHE isoforms (<39 μM), while its Ki for EIPA and HOE694, amiloride analogues, were similar to other colonic NHE isoforms (Table 3). In addition, Cl-NHE activity in crypt AMV isolated from aldosterone-treated rats was 60% greater than that from normal rats. These several observations suggested the identification of a novel NHE isoform. The characteristics of Cl-NHE and other NHE isoforms that are present in colonic epithelial cells are presented in Table 3.

Table 3: Distribution And Kinetics Of Colonic Nhes

	Cl-NHE	NHE1	NHE2	NHE3
Cell distribution	crypt	surface/crypt	surface/crypt	Surface
Membrane distribution	apical	basolateral	apical	Apical
Km for Na (mM)	24.2	15	18	17
Ki for amiloride (μM)	480.2	1-3	1-3	39
Ki for EIPA (μM)	1.1	0.02	1	8
Ki for HOE694 (μM)	9.5	0.3	5	650

Data based on results reported in ref. #4, 16, 45, 46.

To establish the molecular identity of Cl-NHE a cloning strategy was employed that involved RT-PCR using mRNA isolated from crypt cells of normal rat distal colon and primers that were designed based on the J and F membrane spanning domains that are conserved in NHE-1, -2, and -3 isoforms (38). The sequence of one of these 500 bp RT-PCR products had significant homology with a NHE-1 fragment. This cDNA fragment was used to screen a colonic library which yielded a single positive clone that represented a 1.9 kb cDNA consisting of a 700 bp fragment identical to the NHE-1 isoform and 1.2 kb novel fragment with a stop codon within the first 200 bp of the novel sequence. Since a start codon was not identified, 5'RACE was performed resulting in an 800 bp cDNA with a start codon and which overlapped with the previous clone. A full-length cDNA was constructed using primers designed on the 3' and 5' ends. This 2,498 bp full-length cDNA consists of 1,485 bp 5' end that is identical to a fragment of NHE-1 isoform and a 1,013 bp 3' end that is completely novel; this cDNA has a 5' non-coding sequence of 360 bp, a 1,314 bp coding region and a 3' non-coding sequence of 824 bp. The nucleotide sequence of the putative Cl-NHE cDNA encodes a protein of 438 amino acids (375 amino acids that are identical to NHE-1 isoform and 63 amino acids at the C-terminal end) with a calculated molecular weight of approximately 50 kDa.

In situ hybridization studies were performed with a 589 bp novel fragment of Cl-NHE and revealed the presence of mRNA primarily in crypt cells, a pattern consistent with the distribution of Cl-NHE activity (38). Northern blot analyses were also performed with two different Cl-NHE cDNA fragments. When the complete coding region of the putative Cl-NHE cDNA was used as a probe, two mRNA species were identified: a 4.4 and a 2.5 kb transcripts. Using the 589 bp novel fragment northern blot analysis revealed that a 2.5 kb mRNA was present not only in colon, but also in kidney, lung, muscle, testes and brain. These observations are consistent with this cDNA consisting of a NHE-1 isoform fragment and a novel

fragment that has wide tissue distribution. Parallel to the demonstration that Cl-NHE activity was increased by 60% in AMV isolated from crypt cells of aldosterone treated rats (compared to normal control rats), a northern blot analysis of mRNA from distal colon from normal and aldosterone rats using the 589 bp as a probe demonstrated a substantial up-regulation (~ 200%) of the 2.5 kb transcript in aldosterone rats.

Functional studies were performed to establish whether this putative Cl-NHE cDNA manifested Cl-dependent NHE activity (Figure 2). PS120 fibroblasts, a cell line without endogenous NHE activity, were employed for these functional studies of Na-dependent pHi recovery to an acid load induced by an NH_3/NH_4Cl preload. No endogenous NHE activity was present either in the absence or in the presence of Cl (38). In contrast, in PS120 cells that had been stably transfected with the putative Cl-NHE cDNA Na-dependent pHi recovery was demonstrated in the presence, but not in the absence of Cl. This Na/Cl-dependent pHi recovery was inhibited by EIPA, an amiloride analogue that inhibits all NHE isoforms; by NPPB, a non-specific Cl channel blocker; but not by 25 μM HOE694, which at this concentration is an inhibitor of both NHE-1 and NHE-2 isoforms. These studies establish that the Cl-NHE activity expressed in PS120 cells transfected with the putative Cl-NHE cDNA closely resembles the activity in native crypt cells providing excellent evidence that the putative cDNA encodes Cl-NHE. Since NHE-1 is exclusively a basolateral membrane protein, initially it was difficult to conceptualize that C1-NHE that has considerable sequence homology with NHE-1 protein was localized to the apical membrane. It is important, however, to emphasize that colonic H,K-ATPase, that is an exclusive apical membrane protein primarily localized to surface cells, also has a substantial sequence homology with Na,K-ATPase, another obligate basolateral membrane protein. As a result, colonic surface and crypt epithelial cells may have the ability to express modified basolateral membrane proteins on the apical membrane to regulate Na-dependent fluid absorption in either an absorptive or secretory mode. There have been at least three recent examples of Cl-dependent NHE function in non-colonic tissue suggesting that Cl-NHE activity in the colon may not be a unique observation to the colon (39-44). First, Miyata et al identified a Cl component in the adaptive response of NHE to cell shrinkage in rat mesangial cells, but not in the Na-dependent adaptive response to an acid load (39).

Although studies assessing whether there is a Cl-dependent component to the adaptive response to hyperosmolar contraction have not as yet been performed in crypt epithelial cells of rat distal colon, Cl-NHE activity has been identified in association with Na-dependent recovery to an acid load. This suggests that Cl-NHE activity in rat mesangial cells and rat distal colonic crypt cells differ, and probably represent different physiologic

processes. Second, an endogenous NHE isoform was identified in Xenopus oocytes that has partial Cl-dependence (40).

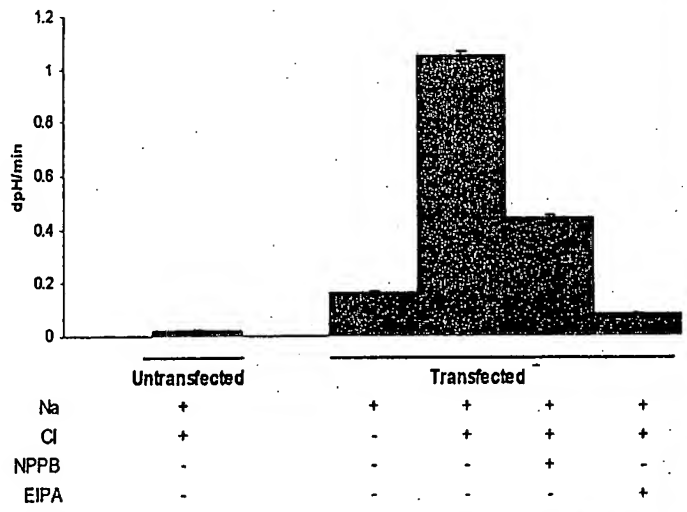

Figure 2: Na-dependent pHi recovery to an acid load in the presence and absence of Cl in untransfected and Cl-NHE cDNA transfected PS120 cells. Significant Cl-independent nor Cl-dependent Na-dependent pHi recovery was seen in the untransfected cells. In contrast, in the Cl-NHE transfected cells, Na-dependent pHi recovery was almost exclusively Cl-dependent and was inhibited both by EIPA, an amiloride analogue, and by NPPB, a Cl channel blocker. These results were adapted from data presented in ref. #38.

Similar to that observed in mesangial cells, NHE activity in response to an acid load manifested only modest Cl-dependence. Third, in NHE-deficient AP-1 cells transfected with NHE-1, NHE-2 or NHE-3 isoforms partial Cl-dependence was also identified (41). However, these investigators did not adequately exclude that the anions, NO_3 and SCN that were used as Cl-substitutes, did not inhibit NHE activity. Nonetheless, although it is likely that evidence of Cl-dependent NHE function may be present more widely than previously appreciated, most of these observations differ from those observed in colonic crypt cells.

Consistent with the widespread distribution of Cl-NHE mRNA in several tissues there are several observations that are suggestive and consistent with a physiological role for Cl-NHE in other tissues; that is, Cl-NHE does not represent a curiosity of rodent colonic epithelial cells. First, Choi et al performed perfusion studies of renal proximal tubule in NHE-2, NHE-3 and NHE-2/NHE-3 knockout mice in the presence of lumen Cl, and Na-dependent proton secretion that was inhibited by EIPA was observed (42).

These findings of an unidentified NHE activity that was neither NHE-2 nor NHE-3 isoform and that represented approximately 50% of total NHE activity in the proximal tubule are consistent with the presence of Cl-NHE. Perfusion studies performed in the absence of lumen Cl were not reported and would be required to assess whether this NHE activity represented Cl-NHE. Second, these investigators also performed perfusion studies of pancreatic ducts in NHE-2, NHE-3 and NHE-2/NHE-3 knockout mice in which Na-dependent HCO_3 absorption (i.e., NHE) was determined. In these studies approximately 55% of total Na-dependent HCO_3 absorption that was inhibited by 50 μM HOE694 was not due to either NHE-2 or NHE-3 isoforms (43). Since these studies were performed in the presence (but not in the absence) of lumen Cl, these observations were consistent with the presence of Cl-NHE in the apical membrane of the pancreatic duct cells. Third, Cl-NHE function was identified in colonic crypt cells obtained from mice and humans in that Na-dependent pHi recovery had an absolute requirement for Cl and was inhibited by NPPB (unpublished observations). Parallel to these observations of Cl-NHE function in mice and humans, Cl-NHE mRNA abundance was also present in northern blot analyses performed with mRNA isolated from mouse and human colon (unpublished observations). In contrast, a recent report did not identify Cl-NHE activity in the cells at the base of the colonic crypt in mice suggesting that Cl-NHE is localized to cells in the mid-crypt (44). The absence of Cl-NHE in the base of the colonic crypt is not surprising in that these cells usually are closely linked to cell proliferation rather than electrolyte transport. Fourth, evidence of Cl-NHE regulation was also observed in that Cl-NHE mRNA abundance was enhanced by aldosterone in rat distal colon and was substantially greater in NHE-3 isoform knockout mice compared to wild-type littermate controls (38). As a result, we suspect that Cl-NHE may be a member of a new class or subfamily of NHE proteins with significant importance in cell homeostasis of multiple tissues and may be the mechanism for one or more Na and Cl transport processes that have not been fully explained by previously identified transport proteins.

3. BASOLATERAL MEMBRANE NHEs

Well established is that NHE-1 isoform is present on the plasma membranes of both non-polar and polar cells. On polar (or epithelial) cells NHE-1 isoform is present on the basolateral membrane and has been often referred to as a "house-keeper" with evidence of its importance in the regulation of cell volume and pHi. The epithelial cells of the mammalian colon originate at the base of the colonic crypt (or gland) and migrate up the crypt over 72-96 hours to become surface cells and then slough. The latter

process representing apoptosis. Although in several tissues and cells NHE-1 isoform has been closely linked to cell proliferation, whether NHE-1 isoform has a similar critical role in cell proliferation in the colon is not known.

In the rat distal colon NHE-1 isoform is present on the basolateral membrane of both surface and crypt cells, and, in contrast to other colonic NHE isoforms (see above), aldosterone does not modify either the activity, protein or message of NHE-1 isoform (Table 2). Na-HCO_3 cotransport is also present on the basolateral membrane of the rat distal colon (47). Limited studies to date indicate that the NBC isoform in the distal colon differs from the NBC isoforms that are present on many other epithelial cells (i.e., renal tubule, pancreatic duct), but that its identity has not been established and that the NBC isoforms in proximal and distal segments of the rat colon are not identical.

Several experimental observations of Na-HCO_3 cotransport in rat distal colon require some mention of colonic NBCs for this discussion of NHE function in colonic epithelial cells. First, in the absence of HCO_3, NBC functions as a NaOH cotransporter which operationally is a Na-H exchanger (47). Second, although traditionally inhibition of Na transport by amiloride indicates the presence of either ENaC or NHE activity, colonic NBC is also inhibited by amiloride (47). Colonic electrogenic Na-HCO_3 cotransport characteristically differs from that in the renal proximal tubules as the former is sensitive to both amiloride and DIDS while the latter is sensitive only to DIDS (48). Thus, it is not unlikely that some prior studies of Na transport that observed amiloride inhibition concluded that a NHE (and not a NBC) isoform was involved.

Recent molecular studies have isolated four different Na-HCO_3 cotransporter (NBC1, NBC2, NBC3 and NBC4) isoforms from one or more tissues (49,50). Expression of presently cloned NBC isoforms exhibit only amiloride-sensitive or DIDS-sensitive Na-HCO_3 cotranport activity, but not sensitivity to both amiloride and DIDS. Consistent with the presence of DIDS-sensitive Na-HCO_3 cotransport activity in renal tubules, expression of NBC1 (that was cloned from kidney) in Xenopus oocytes exhibited DIDS-sensitive Na-HCO_3 cotransport activity. NBC2 and NBC3 are structurally identical except for the insertion of 36 amino acids in NBC3, but their cRNA expression in Xenopus oocytes exhibited quite different Na-HCO_3 cotransport activity: NBC2 expressed DIDS-sensitive activity while NBC3 expressed amiloride-sensitive activity. Limited molecular studies including RT-PCR and northern blot analyses in the rat distal colon demonstrated the expression of NBC3-like, but not NBC1, NBC2 or NBC4 transcripts. As a result, the molecular identity of the colonic NBC isoform(s), which is sensitive to both amiloride and DIDS, is not known.

In contrast, in the rat proximal colon both NBC1-like and NBC3-like transcripts are expressed. Immunocytochemical studies reveal that NBC1-like protein is predominantly expressed in basolateral membranes of mid-

crypt region of rat proximal colon. Uptake studies with basolateral membrane vesicles (BLMV) revealed that the Na-HCO_3 cotransport activity in proximal colon was partially sensitive to amiloride and completely sensitive to DIDS. This pattern of sensitivity to inhibitors suggest the presence of both DIDS-sensitive and DIDS-sensitive/amiloride-sensitive NBC isoforms. These several observations yield the following tentative conclusions about the identity of NBC function in the rat proximal and distal colon: 1) the predominant NBC isoforms in proximal and distal colon are not identical; 2) the NBC isoform in the distal colon has not been identified and is probably not NBC3; and 3) at least two NBC isoforms are present in the proximal colon.

Since experimental data indicates that the NBC activity in BLMV isolated from rat distal colon can function to transport either $NaHCO_3$ or NaOH and since NaOH and Na-H exchange are functionally similar, previous studies with colonic BLMV have examined and compared the regulation of NBC and NHE activity by pH (51). Although increasing proton concentrations stimulate both NBC and NHE activities, the enhancement in these two Na transport processes by protons occur via different kinetic mechanisms. Thus, increasing the intravesicular pH (from 6.5 to 5.5) substantially reduced the Km for Na without altering the Vmax for NHE activity while for NBC activity a similar increase in intravesicular pH enhanced the apparent Vmax without affecting the Km for Na (Figure 3). Further studies established that the magnitude of the pH gradient and not the absolute pH was critical for the regulation of NHE function. NBC activity was regulated by the absolute pH and not by the magnitude of the pH gradient. Additional studies revealed that the increase in NBC activity that is induced by the absolute pH represented an enhanced Vmax without change in the Km for Na. These observations of differential regulation of NHE by pH by distinct and separate mechanisms suggest that these two basolateral Na transport processes regulate different cellular functions. Although these studies determined Na transport across BLMV and did not examine intracolonocyte pHi, based on these observations, we predict that NBC may play an important role in intracellular pH regulation in colonocytes, while NHE may not be required for pHi regulation in these cells, but rather with other cell functions, e.g., cell volume regulation.

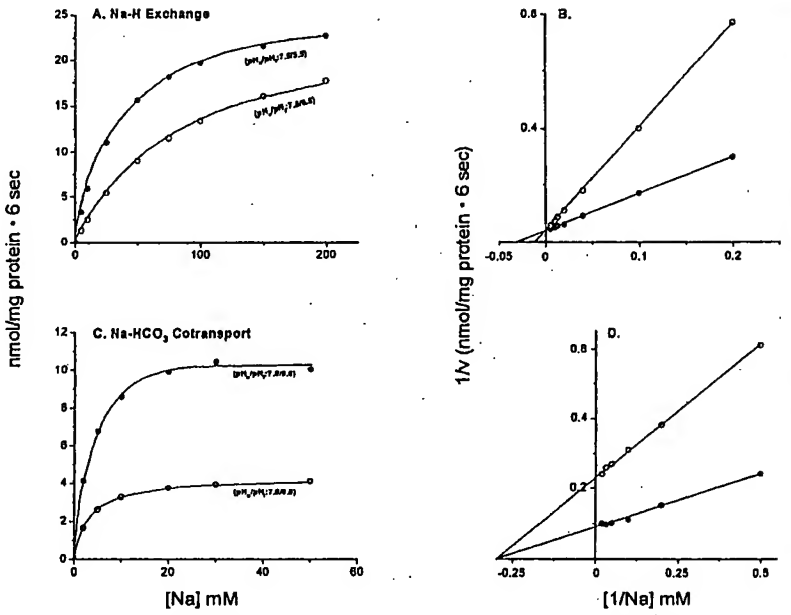

Figure 3: Effect of intravesicular [H] on Na kinetics in BLMV mediated by NHE (A, B: top panels) and by NBC (C, D: bottom panels). Decreasing intravesicular [H] [increasing intravesicular pH (pHi) from 5.5 to 6.5] decreased the affinity for Na (i.e. increased the Km for Na from 35.6 ± 5.4 to 94.4 ± 32.7 mmol/mg .6 sec) without significantly changing the maximal rate (Vmax) of uptake for NHE. In contrast, decreasing intravesicular [H] reduced the Vmax without significant change in Km for Na for NBC. Reproduced from ref. #51.

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Chapter 6

NhaA Na^+/H^+ ANTIPORTER. STRUCTURE, MECHANISM AND FUNCTION IN HOMEOSTASIS OF Na^+ AND pH

Etana Padan, Abraham Rimon, Tzvi Tzubery, Marcus Müller, Katia Herz and Livnat Galili

Division of Microbial and Molecular Ecology, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

1. INTRODUCTION

Every cell, whether prokaryotic or eukaryotic, uses the most common ions, H^+ and Na^+, to store and transduce energy. Since proteins can endure only certain concentrations of H^+ and Na^+, these ions, very often, become very potent stressors to all cells. Therefore every cell has efficient homeostatic mechanisms for Na^+ and H^+ (1). Proteins that play a primary role in these homeostatic mechanisms are Na^+/H^+ antiporters (2).

The Na^+/H^+ antiporters were discovered by Peter Mitchell in the early seventies of the last century (3). In line with their importance in cell physiology, their activity was found in the cytoplasmic membranes of all cells and in membranes of many organelles (2,4) except for one bacterium (5).

In *E. coli* we identified and cloned two genes, *nha*A (6,7) and *nha*B (8) that encode for Na^+/H^+ antiporters. Since then other laboratories cloned antiporter genes from many other eukaryotic and prokaryotic organisms.

Recently the genome project produced a flood of antiporter genes that can already be clustered in families (2).

By knocking out *nha*A (7) and *nha*B (9) in the *E. coli* chromosome, each separately and both together, we found that NhaA is the main Na^+/H^+ antiporter in *E. coli*; it is essential for Na^+/H^+ homeostasis of this organism and many other enterobacteria. NhaB is most probably the housekeeping antiporter that becomes important only when NhaA is absent or not active (9).

Being involved in homeostasis implies certain unique properties for NhaA which will be reviewed here: the transporter itself is equipped with "sensors" to sense the Na^+ and H^+ concentrations of the environment and it has a device to respond to these environmental signals by shutting off or turning on activity, so as to maintain homeostasis.

2. THE RESPONSE TO Na^+ OCCURS AT THE TRANSCRIPTION LEVEL

2.1. Exponential Phase Of Growth

The expression pattern of the reporter gene *lac*Z of a *nha'-'lac*Z fusion was examined in exponentially growing cells. Under various concentrations of Na^+ it showed that, *nha*A responds to Na^+ by increasing transcription via a very intricate novel regulatory system that is highly specific to Na^+. This regulatory system, recently reviewed (1), involves the positive regulator NhaR (10-12) of the LysR family of regulators (13,14), and is induced specifically by Na^+; neither ionic strength nor osmolarity induces the system. Intracellular rather than extracellular Na^+ is the inductive signal (15).

NhaR was purified and its binding site on *nha*A was identified both by gel retardation and foot printing assays. NhaR is always bound to the DNA and Na^+ changes its conformation so that its footprint is Na^+ specific (12).

Primer extension analysis identified two promoters of *nha*A, P1 and P2 (16) of which only P1 is involved in the Na^+ and NhaR dependent regulation of *nha*A (17). P1 is located within the NhaR binding site, and its deletion eliminates Na^+ induction. Accordingly, when fused to the *lac*Z reporter, P1 promotes Na^+ induction of the reporter gene.

2.2 Stationary Phase Cells

*po*S is a sigma factor that in stationary phase bacteria, is recruited to the RNA polymerase to transcribe stationary phase specific genes, the *rpo*S regulon (18-20). Most interestingly, in the stationary phase, *nha*A becomes

part of the stationary phase regulon via p2 (17). when p2 is fused to *lac*Z reporter it promotes induction of *nha*A in the stationary phase. Primer extension shows that this induction is not dependent on Na^+ or NhaR but on RpoS.

3. THE ECOLOGICAL IMPORTANCE OF THE ANTIPORTERS IN THE ENTERIC BACTERIA *ESCHERICHIA COLI* AND *VIBRIO CHOLERAE*

Many enterobacteria similar to *E. coli* and *V. cholerae* share aquatic salinic ecological niches. In these environments they often survive rather than grow. Measuring the survival of *E. coli* mutants showed that expression of *nha*A from the P2 promoter, via the *rpo*S regulon, is essential for the survival of *E. coli* in salinic environment at alkaline pH (17).

We have recently cloned three antiporter genes from *V. cholerae*, Vc-*nha*A, Vc-*nha*B and Vc-*nha*D, the former two are homologous to the respective *E. coli* genes; the latter is homologous to *nha*D of *Vibrio parahaemolyticus* (21,22). Singles, double (in all combinations) and triple mutants were constructed in *V. cholerae*. In contrast to *E. coli*, where a mutant inactivated in the two antiporter genes, *nhaA* and *nhaB*, is more susceptible to NaCl than each of the respective single mutants (9), the inactivation of all three putative antiporters (Vc-*nha*ABD) did not alter the exponential growth of *V. cholerae* in the presence of high Na^+ concentrations and had only a slight effect on the bacterial survival in the stationary phase. In contrast, a pronounced and a similar Li^+ sensitive phenotype was found with all mutants lacking Vc-*nha*A during the exponential phase of growth and a marked decrease in survival of the triple mutant was observed in the stationary phase in the presence of Li^+ (22).

Similar to *V. alginolyticus, V. cholerae* possesses (Fig. 1) an electron transport-linked Na^+ pump, the NADH-quinone oxidoreductase complex (NQR) (23-25). The NQR pump extrudes specifically Na^+ but not Li^+ (23). This Na^+ specific activity of the pump, may explain the higher contribution of the *V. cholerae* Na^+/H^+ antiporters to Li^+ resistance as compared to Na^+ resistance; in the absence of Na^+/H^+ antiporters, the NQR pump can compensate for the Na^+/H^+ but not the Li^+/H^+ antiport activity, resulting in a Li^+ sensitive but not a Na^+ sensitive phenotype.

To test this possibility, we used NQNO (NQNO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide), a quinone analogue similar to that previously shown to inhibit the NQR from *V. alginolyticus* (23). The results show that 25 μM NQNO, have no effect on the growth of wild type *V. cholerae*. However, as little as 12.5 μM NQNO inhibited dramatically and to the same extent the growth of both Vc-*nha*A *and* Vc-*nha*ABD mutants (22). These results strongly suggest that NhaA is involved in the Na^+ and H^+ homeostasis

of *V. cholerae* at alkaline conditions but, its contribution can only be revealed when the Na^+ pump activity of NQR is inhibited (Fig. 1). Our results show that to understand the Na^+ resistance of the pathogen *V. cholerae*, it is essential to study the inter-relationship between the Na^+/H^+ antiporters and the NQR Na^+ pump, both contributing to the Na^+ cycle of *V. cholerae*.

Recently it was shown that, in *E. coli* and in other enterobacteria, complex I (NDH I) of the electron transport chain extrudes Na^+ (26). We therefore suggest that *E. coli* and *V. cholerae* represent two modes of adaptation to high salinity at alkaline pH in enteric bacteria, in which NhaA is the prominent antiporter (Fig. 1); While in *E.coli*, NhaA appears to be the dominant factor in this adaptation (1), in *V. cholerae* it functions with NQR. Further studies are needed to clarify the contribution of each Na^+ translocation system to the Na^+ cycle of these bacteria. Interestingly, the Na^+ cycle plays a role (in a mechanism which is not yet understood), in the virulence of *V. cholerae* (27).

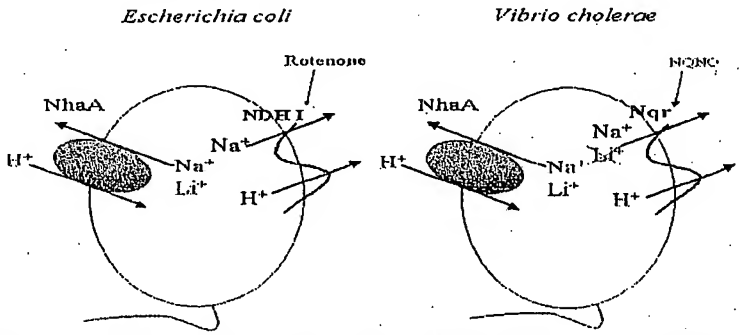

Figure 1. The two modes of Na^+ cycle in enterobacteria. Nqr, NADH-quinone oxidoreductase (23,24); NQNO, (NQNO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide); NDH I, complex I of the respiratory chain (26).

4. THE NhaA PROTEIN

A critical step in the study of NhaA protein was the construction of a most efficient over expression system for *nhaA* (28-30). In this system, NhaA fused to 6 histidines at its C-terminus, is expressed from the strong NhaA fused to 6 histidines at its C-terminus, is expressed from the strong and regulated *tac* promoter. The *tac* promoter allows for very strong over expression and the His-tag for a very efficient purification of the protein product on Ni-NTA affinity column, in amounts as high as 5 mg/L of cells and as concentrated as 40 mg/ml (29).

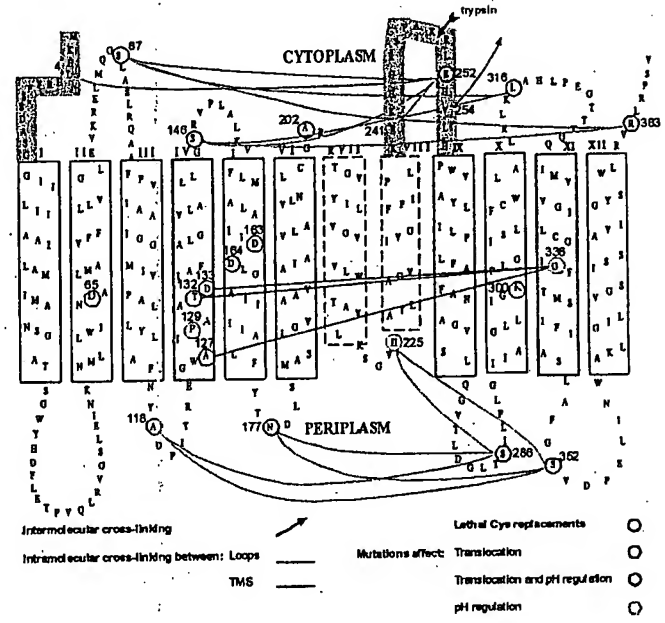

Figure 2. Two D model of NhaA (31). Positions where amino acid replacements cross-link (37), affect translocation (40) or pH regulation (40, 45, 46,47) are indicated. Domains that change conformation with pH are shown with dark back ground.

When reconstituted in proteoliposomes, the purified protein is fully active and shows the native characteristics of NhaA observed in membrane vesicles (reviewed in (4)): it exchanges $2H^+/1Na^+$ and is tightly regulated by pH. This high yield of purified active NhaA opened the way to structural studies, as yet a pioneering venture in membrane proteins.

4.1. The Structure Of NhaA, 2D Crystals

A two-dimensional model of NhaA protein was predicted from the amino acid sequence ((31) and Fig. 2). As many other transporters it is composed of 12 transmembrane segments. This model was verified in various ways including *pho*A fusions (31), determination ofaccessibility of specific residues to various agents from either the cytoplasmic or periplasmic side of the membrane and antibody epitope mapping (28,31).

Most importantly recently we obtained a direct proof for the model: 2D crystals that diffract electrons at 4 Å were obtained (32). This provided a projection map (32) and a tri-dimensional reconstructed map of NhaA ((33) and Fig 3A).

In the crystal, NhaA forms a dimer in which each monomer is composed of 12 helices. Helix assignment is in progress. This was the first insight into the architecture of an ion-coupled polytopic transport protein.

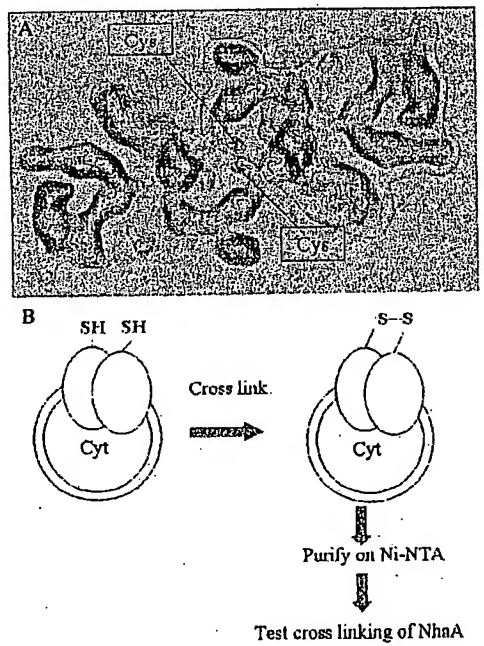

Figure 3. Intermolecular cross-linking between NhaA monomers. 3A, The projection map of NhaA 2D crystals (32) is shown with arrows, pointing at twin domains, with Cys replacements, one per molecule. 3B, A protocol for the intermolecular cross linking of NhaA. The affinity purified protein is analyzed on SDS-PAGE for monomers and dimers (34)

4.2. On The Way To Atomic Resolution Of NhaA, 3D Crystals

NhaA readily crystallizes in 3D, yielding huge and hollow-inside crystals. Although these crystals diffract X rays poorly, they broke the dogma that polytopic membrane transport proteins do not crystallize.

To improve diffraction, the approach advanced by H. Michel has been undertaken (35): co-crystallize NhaA with monoclonal antibody (mAb) fragments (FV or FAB) of anti-NhaA, conformational specific, mAbs. The Fv provides hydrophylic surface to form crystal contacts. It also fixes one conformation of the protein, as a result increasing the homogeneity of the protein preparation, an essential requisite for crystallization. Four mAbs, NhaA specific, were isolated: three recognize only native epitopes (36), are Western negative, but bind native NhaA, as observed on sizing column in HPLC; three inhibit the antiport activity. We have already obtained co-crystals of NhaA with Fv of mAb 2C5 or FAB 2C5. While the former diffract X rays to 7-8 Å the latter diffract to 6 Å resolution.

4.3. NhaA Is A Dimer In The Membrane

As shown above NhaA is a dimer in the 2D crystals (32,33). It is also a dimer in solution as determined by HPLC (36). Does the dimer exist in the membrane or does it just reflect a favorable crystalographic interaction with no relation to activity?

To test physical interaction between NhaA monomers within the membrane, His-tagged NhaA was co-expressed with HA (hemagglutinin)-tagged NhaA. Purification on Ni-NTA showed that the HA-tagged NhaA co-purified with the His-tagged - NhaA, clearly showing, that, within the membrane, physical interaction exists between NhaA monomers (34).

4.4 Mapping The Interface Between NhaA Monomers

The 2D crystal of NhaA, revealed that, in the dimer, the contact area between the monomers is very narrow and twin helices, one (at least) from each monomer, form the interface (Fig. 3A). To map the interface, single cysteines, one per each monomer, were introduced to NhaA loops. A Cys introduced to a loop, that is involved in the interface, should be able to cross-link with its twin Cys of the other monomer (Fig. 3A and B). Screening all loops with three different cross linking agents (BMH, 1,6-bis-(maleimido)hexane; *o*-PDM, *N,N-o*-phenylenedimaleimide and *p*-PDM, *N,N-p*-phenylenedimaleimide) showed that loop VIII-IX resides in the interface between the monomers ((34) and Fig. 2).

4.5. Proximities Between Helices Of NhaA

Whereas NhaA has many potential trypsin-cleavable sites in loops, none are accessible at neutral or acidic pH and only one site, K249, is accessible at alkaline pH (see section 6.1.1., Fig. 2 and 37). This unique trypsin cleavable site was exploited to detect a change in mobility of cross-linked products of NhaA on SDS-PAGE (Fig 4 and (37)). Double Cys replacements were introduced into loops (Fig. 2 and (37)), one on each side of the trypsin cleavage site (K249). The proximity of paired Cys residues was assessed by disulfide cross-linking of the two tryptic fragments, using three homobifunctional cross-linking agents, BMH, o-PDM and p-PDM and the distances between the double Cys-replacements were assessed.

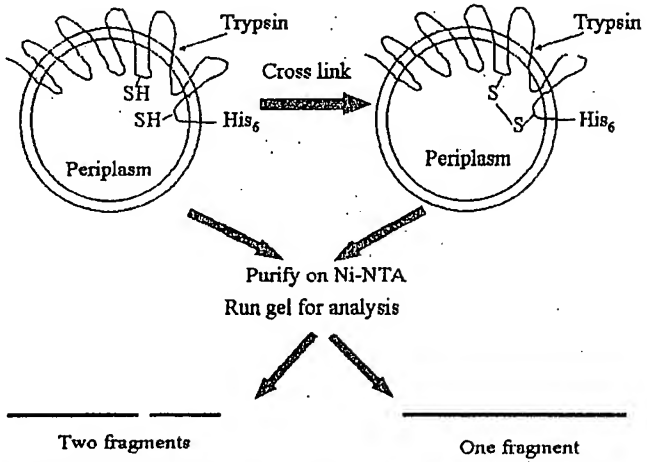

Figure 4. Intra molecular cross linking of NhaA. The protocol (37) is schematically shown. For further explanation see text.

The inter-loop cross-linking was found to be very specific, a conclusion consistent with the notion that, loops as helices are not merely flexible polypeptides that interact randomly. In the periplasmic side of NhaA two patterns of cross-linking are observed (Fig.2): a) all three cross-linking reagents cross-link very efficiently between the double Cys replacements A118C/S286C, N177C/S352C and H225C/S352C. b) only BMH cross-links the double Cys replacements A118C/S352C, N177C/S286C and H225C/S286C. In the cytoplasmic side of NhaA three patterns of cross-linking are observed (Fig. 2and Fig. 5): a) all three cross-linking reagents cross-link very efficiently the pairs of Cys replacements L4C/E252C, S146C/L316C, S146C/R383C and E241C/E252C; b) BMH and p-PDM cross-link efficiently the pairs of Cys replacements, S87C/E252C, S87C/L316C, S146C/E252C: c) none of the reagents cross-links the double Cys replacements, L4C/L316C, L4C/R383C, S87C/R383C, A202C/E252C, A202C/L316C, A202C/R383C, E241C/L316C and E241C/R383C. Remarkably, the data reveal that the N-terminus and loop VIII-IX that have previously been shown to change conformation with pH, are in close proximity within the NhaA protein (see section 6.1.1., 6.1.2., 7.1. and Fig. 5). The data also suggest close proximity between N-terminal and C-terminal helices at both the cytoplasmic and periplasmic face of NhaA.

Hence, although as yet we do not have atomic resolution, the low resolution structure of NhaA obtained from the 2D crystals combined with the cross-linking data already provides an intriguing basis to study the function of NhaA in relation to its structure.

5. RELATIONSHIP BETWEEN FUNCTION AND STRUCTURE OF NhaA.

5.1. The pH Response Of NhaA Occurs At The Protein Level

When reconstituted in proteoliposomes, pure NhaA on its own exhibits the pH response (4,38,39). It is shut off below pH 6.5 and changes its Vmax dramatically by over three orders of magnitude upon shift to alkaline pH, reaching maximum at pH 8.5. Hence NhaA is equipped with a pH sensor and a device that transduces the pH signal into a change in activity. The question arises as to whether the amino acid residues involved in the pH response are identical, overlap or different from the ones that are involved in the translocation of the ions.

5.2. Differentiating Between Residues Involved in the Translocation Pathway and the pH Regulation

Mutagenesis, site-directed and random, are most important tools that have to be combined to structural studies to identify residues that are involved in the activity of a protein. To identify residues involved in ion translocation, site directed mutagenesis of amino acid residues that have the chemical capacity to attract, bind or repel cations was performed (Fig. 2). NhaA has 4 Asp and 2 Lys in the helices. These were mutated either to Cys (40) or Asn (41,42). Another approach, based on the notion that nature repeats its successes, is to mutate residues that in other proteins have been

shown to bind Na^+. A model of the Na^+ binding site of a Na^+/ATPase has been developed (43,44). In this model hydrophilic residues such as threonine or asparagine neighboring aspartates were found to be involved. N64 and T132 were therefore mutated to Cys (40); finally random mutagenesis was applied on plasmidic *nha*A and mutants which change the specificity or affinity to the ions were selected (40,42).

To identify residues involved in the pH response, site directed mutagenesis was performed on residues having pK in the physiological range (Fig. 2). Therefore all His were mutated to Arg (45-47). We also applied random mutagenesis of plasmidic *nha*A and selected mutants which affect the pH profile; grow in the presence of Na^+ at neutral pH but not at alkaline pH (47).

To characterize the mutants it was essential to differentiate between residues affecting the translocation, the pH response or both. On each mutant therefore a kinetic analysis was performed at various pHs (40). Mutants that affect the Km only, when assayed at saturating concentrations of the ions, should not show any shift in the pH profile. In contrast, mutants that affect both parameters or the pH response only, exhibit a change in the pH profile even at saturating concentrations of the ions.

5.2.1. Mutants Within Putative α Helices

Four types of mutants were identified within helices (Fig. 2): 1) D163C, D164C and K300C - proteins are fully expressed, with no antiporter activity (40,41). 2) D65C, D133C and T132C affect dramatically the Km of the transporter to both Na^+ and Li^+ with no effect on the pH profile (40). We suggest that the residues mutated in the latter two groups are involved in the Na^+ translocation pathway. Although D133C and D164C are in the middle of α helices, they are accessible to impermeant SH reagents in inverted membrane vesicles but not in right side out vesicles (40). 3) A127V, affects both the pH profile and the translocation pathway (40). 4) G338S, affects only the pH dependence of NhaA (47).

Mutants such as G338S are pH conditional lethal mutants (47); they do not grow at alkaline pH in the presence of Na^+. Suppressor mutations selected by growth of G338S at alkaline pH in the presence of Na^+ were found clustered in helix IV (Fig. 2). These include: A127T, P129L and A127V. It is most interesting that A127V has also been obtained by selecting for mutants that cannot grow in the presence of Li^+ (see above and (40)). The mutations in helix IV that suppress mutants in helix XI, highly suggest that these two helices are in close proximity and possibly functionally interact (37).

All these mutated residues that affect the translocation or the pH response are conserved (40). Taken together these results show that the residues that affect the Km may differ from those that affect the pH profile.

5.2.2. Mutants Within Loops

As yet, mutants that drastically affect the pH response only, have been found in loops (Fig. 2): in the N-terminus, L4C (48); in loop VII-VIII, H225R (45); in loopVIII-IX, V254C and E241C (34). It should be noted that loop VIII-IX is located in the interface between NhaA monomers (see section 4.4. and (34)).

6. DYNAMICS OF NhaA.

6.1. The pH Signal Is Transduced In The Protein By A pH Induced Conformational Change

6.1.1. Identifying Conformational Changes By Accessibility To Trypsin

In many proteins accessibility to trypsin has been used to look for a change in conformation of a protein in response to various signals, ligands or conditions. NhaA has many potential trypsin cleavable sites in its loops (49). Exposure of isolated inverted membranes vesicles, expressing S^{35}-methionine labeled NhaA, to trypsin at various pHs, showed that NhaA is accessible to cleavage at alkaline pH but completely resistant at acidic pH (49). The pH profile of the accessibility to trypsin is similar to the pH response, suggesting that the pH profile of trypsin cleavage reflects a conformational change that is required for activity.

Remarkably, the purified protein solubilized in detergent (dodecyl maltoside) showed the same behavior (50); cleavage at alkaline pH and resistance at acidic pH. Only two tryptic fragments were obtained indicating that only one cleavage site becomes available to trypsin with pH. The fragments were isolated and their N-terminus sequence was determined, identifying K249 as the cleavage site ((50) and Fig. 2). In the native membrane K249 is also the only trypsin cleavage site. Hence loop VIII-IX, in which resides K249, changes its conformation with pH. Is this loop involved in the pH response? Yes, since as we described above mutants V254C and E241C change dramatically the pH response (34).

Recently a *nha*A homologue was cloned from *Helicobacter pilorus* (51), HP-*nha*A and expressed in *E. coli*. HP-NhaA is much less dependent on pH

as compared to *E. coli*-NhaA; in line with the native acidic environment of *H. pilorus*, HP-NhaA is active both at acidic and alkaline pH. The two NhaAs are very similar, but differ in the N-terminus and loop VIII-IX (2,51).

6.1.2. Identifying Conformational Changes By The Use Of mAbs

Another approach to identify conformational changes is the use of monoclonal antibodies that recognize native epitopes. We have isolated 4 mAbs against native conformations of NhaA (36 and section 4.2.). One, 1F6, showed unique properties. It bound NhaA in dodecyl maltoside detergent at alkaline pH but not at acidic pH, as shown on gel filtration HPLC (36,48). The epitope of 1F6 mAb on NhaA was mapped at the N-terminus (48). Remarkably the pH dependent binding of mAb 1F6 reflects the pH dependent active conformation of NhaA. Thus G338S, a mutant that lost pH regulation (47) binds mAb 1F6 in a pH independent fashion(48).

Does the N-terminus involve in the pH response? Yes, since mutation L4C described above and (48) affects the pH profile of NhaA.

7. DYNAMICS IN 3D OF THE pH INDUCED CONFORMATIONAL CHANGES OF NhaA

As shown above two loops of NhaA undergo a dramatic pH induced conformational change (48,50). One of these is loop VIII-IX that resides in the interface within NhaA dimer (34), raising the question whether the interaction between monomers is important for the pH response. The other pH responsive domain is the N-terminus. Since both loop VIII-IX and the N-terminus are cytoplasmic, the question of a pH dependent interaction between loop VIII-IX and the N-terminus is also raised.

7.1. The pH Induced Conformational Change Of Loop VIII-IX Involves The Interface Between NhaA Monomers

Loop VIII-IX changes its conformation with pH (50) and contains residues that are involved in the pH response (50) This loop is located in the interface between NhaA monomers (34). Most interestingly, cross-linking between these loops of the NhaA dimer with a rigid and short cross-linking agent caused a dramatic change in the pH response as opposed to no effect of a long and flexible agent (34). It is concluded that there is a functional interaction between NhaA monomers pertaining to the pH response.

A most powerful approach to test functional interaction between monomers is the genetic approach. In this approach two lethal mutants are co-expressed. If functional complementation is obtained the polypeptides must interact *in vivo*. Functional complementation was indeed found between lethal pH mutants H225R and G338S (34).

It is remarkable that in the putative helix packing of NhaA, the domains that change conformation with pH, the N-terminus and loop VIII-IX are in very close proximity (Fig. 5).

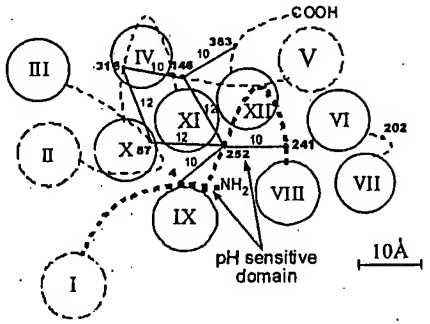

Figure 5. Schematic presentation of helix packing of NhaA at the cytoplasmic face of the membrane. Distances in angstroms estimated from cross-linking between two Cys replacements (37) are shown. TMS are depicted as large circles with Roman numerals from I to XII. Broken circles designate large uncertainty in the spatial positioning of the respective TMS. Loops are presented as dashed lines in which each dash represents amino acid. The figure shows that the two domains of NhaA, that change conformation with pH, the N-terminus and loop VIII-IX are within very close proximity (37).

8. CONCLUSION

Na^+/H^+ antiporters are polytopic membrane proteins that play primary role in pH and Na^+ homeostasis of cells throughout the biological kingdom.The plethora of antiporter genes has recently been enlarged by the genome project, revealing that the Na^+/H^+ antiporter proteins cluster in several protein families. The key antiporter in *Escherichia coli* and other enterobacteria is NhaA. However whereas in *E.coli* NhaA is the dominant factor in *Vibrio cholera* it functions with the primary Na^+ pump NQR. The importance of the antiporters in the virulence of these bacteria is still an open important question. In *E.coli* the transcription of NhaA is regulated by an intricate regulatory system which in the logarithmic phase is dependent on Na^+ and NhaR, a positive regulator and in the stationary phase on RpoS in a Na^+ independent fashion. Over-expression, purification and functional reconstitution of NhaA in proteoliposomes paved the way to structure and

function studies. 2D crystals were obtained and analysed by cryo-electron microscopy providing a density map at 4 Å resolution and 3D structure reconstruction, the first insight into the architecture of polytopic membrane protein. It was revealed that NhaA is a dimer of monomers, each composed of 12 transmembrane segments with an asymmetric helix packing. Genetic and biochemical studies showed that also in the membrane NhaA is a dimmer. Corroborating its role in pH homeostasis, NhaA as many other Na^+/H^+ antiporters are regulated directly by pH. The molecular mechanism underling the pH regulation has been thoroughly studied with NhaA. It was found that amino acids residues involved in the pH response either overlap or not with those involved in the translocation process. Conformational changes transducing the pH signal into change in activity were found in loop VIII-IX and at the N terminus. Remarkably in helix packing model based on cross-linking study the two pH sensitive domains were found in close proximity.

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Chapter 7

THE USE OF TRANSGENIC ANIMAL MODELS TO STUDY Na^+/H^+ EXCHANGE

Larry Fliegel[1] and Morris Karmazyn[2]
[1]Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, and [2]Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada

1. INTRODUCTION

Na^+/H^+ exchangers are a family of membrane proteins that regulate intracellular pH. The plasma membrane isoforms remove an intracellular proton, in exchange for an extracellular sodium. There are seven characterized isoforms of the protein (NHE1-NHE7). NHE1 is virtually ubiquitous in distribution (1-3). NHE2 and NHE3 are predominantly located in the apical membrane of epithelia with NHE2 being found in larger amounts in the stomach, and intestine (4, 5). NHE-3 is expressed at high levels in the colon and small intestine and is also present in the kidney and stomach (6). A significant fraction of NHE3 is also present intracellularly in recycling endosomes (7). While many of the general physiological functions of the isoforms are obvious from their activity, distribution and localization, many of their more subtle functions are difficult to discern especially in intact animals. Some of the Na^+/H^+ exchangers have roles in development and differentiation (See chapter 14 of this text) and it is often difficult to precisely determine what these are unless intact animal models are used. To further complicate the understanding these proteins, membrane proteins are notoriously difficult to overexpress in sufficient amounts to allow their purification. This has held back structural analysis of Na^+/H^+ exchangers with the exception of the prokaryotic Na^+/H^+ exchangers that are more easily expressed in bacteria (3).

One approach to the problem of understanding the structure and function of Na^+/H^+ exchangers has been the transgenic approach. The ability to generate mice with deletions, with overexpression of a gene, or targeted mutations of desired genes has been a great advance in understanding the function of gene products in healthy and diseased animals. Knockout mice can be models of human genetic diseases while overexpression of genes can either duplicate or mimic specific diseases. The ability to specifically target and replace a gene with a mutated one allows whole animal models to be made that can duplicate genetic diseases. In addition it is possible to link known promoters of genes to easily detectable reporter groups and make transgenic mice that allow the analysis of gene expression.

A major benefit of transgenic technology is that it allows the physiological analysis of the protein function in whole animal models *in vivo*. It is possible to determine the function of a gene or mutant gene in the normal resting state of the animal or in different disease states induced in the animals such as a heart disease model. The effect on development of intact animals can be monitored and it is possible to target expression or overexpression of a mutant or wild type gene to a specific organ.

Armed with this battery of experimental techniques, some investigators have begun transgenic approaches to study the Na^+/H^+ exchanger family. Transgenic approaches have been used to study the NHE1, NHE2 and NHE3 isoforms. In addition to direct approaches that knock out these genes, there has been analysis of a natural knockout of the NHE1 isoform, preliminary studies of NHE1 overexpressors and related studies on transgenic mice that have mutations affecting Na^+/H^+ exchanger regulation. There has also been an analysis of the developmental pattern of expression of the NHE1 isoform. The purpose of this chapter is to review the recent results that have been obtained using transgenic approaches to study the Na^+/H^+ exchangers.

2. TRANSGENIC STUDIES ON THE NHE1 ISOFORM OF THE Na^+/H^+ EXCHANGER

2.1 Studies On NHE1 Deficient Mice

The first study of an NHE1 deficient mouse came about as the result of a spontaneous mutation in a colony of mice. Selective breeding and screening determined that the NHE1 locus was the gene affected (8). The mutant localized to chromosome 4 in mice and Northern blot analysis determined that mice homozygous for the defect expressed no NHE1 mRNA while Western blot analysis confirmed the absence of the protein. The genetic defect was a change to a stop codon of lys 442. Cell lines from homozygote

mutant mice showed no significant Na^+/H^+ exchange activity in comparison to wild type cells that had normal activity.

The most noticeable phenotype of the mice was that they had a slow-wave epilepsy mutation. In concert with this defect, they had an ataxic gait including locomoter ataxia, that was most prominent in hind limbs. The mice exhibited periods of behavioral arrest. These behavioral symptoms could be explained at least in part by obvious neuronal defects. Serial histological sections from the brains of affected mice revealed progressive neuronal degeneration of the cerebellum in the deep nuclei at 3 weeks of age. This worsened with age and severity of the disease. There were other significant abnormalities in growth and development. The mutant mice were smaller in size than wild type. Less than half of the homozygous mutant animals survived to weaning and most died by age of 35-40 days. However, no structural abnormalities were seen in organs that were examined by histology other than neuronal abnormalities (8).

This study (8) was the first examination of the effect of absence of a Na^+/H^+ exchanger. It was surprising initially that the mice survived considering the multifaceted role that this protein plays in so many physiological events (9). Within this paper (8) they noted that mice with an absence of NHE1 exhibited reduced growth rates and survival. However this phenomenon was not well characterized. More recently Bell et al. (10) characterized the effects of targeted disruption of NHE1 on growth and survival in more detail. The disruption of the NHE1 gene was designed such that amino acids 217-275 would be replaced - this mutation ensuring that more of the membrane domain was not expressed. The effectiveness of the gene disruption was confirmed by examining cells from homozygous mice with disrupted NHE1. These did not show any Na^+/H^+ exchanger activity. Of the mutant homozygous knockouts, 68% died prior to postnatal day 29. It was found that newborn pups were noticeably smaller than wild type by 2 weeks of age. Homozygous mutants could not mate with other homozygote mutants and carry a litter to term, however matings with wild type and heterozygotes could be successful. Mutant mice also displayed ataxic gait and seizure like behavior similar to the previously described NHE1 deficient mice. Other defects also noted in these mice were accumulations of waxy materials on the surface of paws, inside of ears, and around eyes and chin. In addition they found abnormalities in the stomach of homozygotes where the epithelium from the muscularis mucosa to lumen was thinner in the mutant mice. A widening of the interstitial space between gastric glands was observed. Surprisingly, there was no effect on blood gases and plasma electrolytes. It was suggested in this and the first study, that the genetic background of the mice affected the severity of the defect in the mice.

By the creation of Na^+/H^+ exchanger null mice, it was thus possible to examine the function of Na^+/H^+ exchangers in several cell types and compare the knock vs. wild type cells. Na^+/H^+ exchanger activity is believed to be an important part of the response to muscarinic stimulation by salivary glands. An acid load is created in the response to a secretion stimulus of mouse parotid acinar cells. To examine which Na^+/H^+ exchanger isoform is important in this response one group (11, 12) examined the intracellular pH recovery from parotid acinar cells of mice with targeted disruption of either NHE1, NHE2 or NHE3. pH regulation was only defective in mice with a defect in NHE1. The defect occurred in bicarbonate containing or bicarbonate free medium. These results were a direct method of determining which Na^+/H^+ exchanger isoform is responsible for pH regulation in these cells. The same approach was used to examine which Na^+/H^+ exchanger was important in sublingual acinar cells. The recovery from acid load was examined in cells from NHE1 and NHE2 knockout mice. Only disruption of the NHE1 isoform of the Na^+/H^+ exchanger caused a defect in pH recovery in response to acid load (13). Therefore, using this type of approach, it was possible to isolate the physiological role of these isoforms in these cell types. It was also possible to ascertain that Na^+/H^+ exchange was responsible for pH regulation as opposed to Na^+-HCO_3^- transport. For example, in NHE1-deficient mice, there was no recovery in response to an acid load even in the presence of HCO_3^-(13). The same type of general strategy was used to examine the role of the NHE1 isoform of the Na^+/H^+ exchanger in O_2 deprivation in mouse CA1 neurons. These neurons exhibit a rapid rise in intracellular pH when subjected to O_2 deprivation. HOE-694 (a Na^+/H^+ exchanger inhibitor with high specificity towards NHE1) eliminated this effect however, when examining CA1 neurons from NHE1 null mice, the effect was only reduced in amount and was not eliminated. Thus other isoforms of the Na^+/H^+ exchanger were implicated in this physiological response (14). Overall it is clear that this transgenic approach can be used to determine which general type of pH regulatory protein is important to a physiological process.

To elucidate the mechanisms by which the absence of NHE1 results in neurological defects, some authors have studied pH regulation and excitability in CA1 neurons of normal and NHE1 nil mice. Hippocampal CA1 neurons of mutant mice have a much higher excitability than in wild type mice that is caused at least partly on upregulation of Na^+ current density (15). These results demonstrated that the lack of NHE1 affects other aspects of nerve cell function not only through Na^+ and H^+ exchange. It was suggested that the absence of NHE1 protein disturbs interactions of specific proteins that form a functional complex in the neuronal membrane. Further study of CA1 neurons in the NHE1 mutant mice showed that pH regulation

is defective in these cells. Most surprisingly, the defect was exaggerated in the presence of HCO_3^-(16). These studies suggested that there was a link between the NHE1 isoform of the Na^+/H^+ exchanger and HCO_3^- dependent and independent transporters. Recently, we have shown that such a link may in fact occur through an interaction with carbonic anhydrase, supporting these observations (17).

2.2 Studies Overexpressing the NHE1 Isoform Of The Na^+/H^+ Exchanger

One group has examined the effect of overexpression of the Na^+/H^+ exchanger in transgenic mice. The rabbit NHE1 gene was expressed under the control of human elongation factor 1α promoter and this gave a widespread but varying level of expression between tissues. Since elevation of Na^+/H^+ exchanger activity is a well known phenotype of essential hypertension (18, 19) they examined these transgenic mice overexpressing in renal tubules. Excretion of water and Na^+ was impaired in the mice and systolic blood pressure was elevated after salt loading. It was suggested that overexpression of the Na^+/H^+ exchanger in apical membranes could result in excessive Na^+ reabsorption through renal tubular cells. Further analysis of these mice has shown that caffeine induced contracture of vascular smooth muscle cells was greater than that of controls and intracellular calcium levels were elevated (20). These results were interesting and provide some insights into mechanisms of hypertension. However it should be noted that genetic linkage analysis and sequence analysis (21, 22) have excluded the Na^+/H^+ exchanger gene as a candidate gene in human essential hypertension. It may be that posttranslational regulation of the protein increases activity of the protein seen in various hypertensive models. In this regard, phosphorylation of the Na^+/H^+ exchanger has been shown to be elevated in hypertension (23, 24) possibly accounting for its increased activity. The transgenic model of hypertension may nevertheless provide useful insights into the role the Na^+/H^+ exchanger plays in this disease.

2.3 Transgenic Approaches To Study NHE1 Regulation

We recently used a different kind of transgenic approach to study regulation of expression of the NHE1 isoform of the Na^+/H^+ exchanger. Transgenic mice were made with the NHE1 promoter driving expression of a reporter gene, either green fluorescent protein or ß-galactosidase (25, 26). The purpose of creating these mice was to use the reporter to examine

developmental and tissue specific expression of the protein. The NHE1 promoter activity could be examined by studying reporter expression. These studies led to further insights into the expression of NHE1. Transcriptional activity varied greatly during embryonic development. The heart and liver showed the greatest levels of expression during embryonic development of 12- to 15- day old mice. Transcription from the NHE1 promoter declined with age. The 15-day old mice had less transcription than 12-day old mice, while in 1-day-old neonates expression of the reporter was not detectable above background levels. In the heart, we were able to show that the NHE1 promoter drove expression of the reporter in myocyte cells (26). The advantage of using this transgenic technique is several fold. Firstly, it allowed examination of transcription in embryonic mice. This would be difficult to do by other methods because of the small amounts of tissue available. PCR based methods are difficult and would only reveal mRNA levels (and not transcription rates) while quantification by immunocytochemical methods is often inaccurate and hampered by lack of good quality antibodies that can adequately detect the low level of NHE1 protein.

Rather than alter the expression of the NHE1 protein, a different transgenic-based approach is to alter putative regulatory or associated proteins. Chronic β_1-receptor stimulation of the heart results in cardiomyocyte hypertrophy, left ventricular fibrosis and heart failure. One model of chronic B1 receptor stimulation is cardiac specific overexpression of the β_1-adrenergic receptor. These mice develop heart failure with the symptoms of β_1-receptor stimulation (27). Since Na^+/H^+ exchange had earlier been shown to be a causative factor in some kinds of hypertrophy (28) this study examined the role of NHE1 in this model of hypertrophy. Transgenic mice showed significant hypertrophy when expressing the β_1--adrenergic receptor and this was associated with large increases in NHE1 messenger RNA and significant increases in the protein levels. It was possible to block the detrimental effects of β_1-adrenergic receptor expression by using the Na^+/H^+ exchanger inhibitor cariporide. Cariporide effectively prevented hypertrophy, fibrosis, and loss of contractile function that occurred in the transgenic mice. These results demonstrated the importance of the Na^+/H^+ exchanger in hypertrophy in a transgenic system that mimics clinical heart failures which occur in patients with elevated plasma catecholamine levels (29, 30).

We recently used a similar transgenic approach to study regulation of expression of the Na^+/H^+ exchanger. The transcription factors AP-2 and COUP-TF I and II have been shown to be important in regulation of the NHE1 promoter in intact cells in culture (31, 32). To examine the role of specific isoforms of these transcription factors in regulation of expression of

the NHE1 isoform we used transgenic mice. The mice had knockouts of the transcription factor AP-2α and COUP-TF1. In mice of embryonic day 18, no difference in Na^+/H^+ exchanger protein expression was detected in heart, lung, liver, kidney and brain from animals heterozygous for the AP-2α deletion. Interestingly, there was an increase in NHE1 protein expression in the brain of an 18-day old embryo homozygous for the AP-2 disruption (26). These results suggested that another member of the AP-2 transcription factor family may be responsible for activation of the NHE1 gene. To date, three proteins are in this family: AP-2α, AP- 2β, and AP-2γ (33) and these results suggested that the AP-2α isoform is not critical for expression.

We found similar results with the transcription factor COUP-TF1 (26). We examined NHE1 protein levels in various organs from 18-day old embryos of a COUP-TF1 knockout line. There were no differences in the quantity of NHE1 protein in organs from animals lacking COUP-TF1 compared to wild type embryos. These results showed that COUP-TF1 may act on the NHE1 promoter at a different stage of development or that another member of the chicken ovalbumin upstream promoter family of transcription factors (ie COUP-TF11). In fact we earlier found that COUP-TFII was more effective in enhancing Na^+/H^+ exchanger expression than COUP-TFI (32). These results suggest that it is more significant in regulating Na^+/H^+ exchanger transcription in intact animals than COUP-TF1. Since deletion of COUP-TFII results in death of embryos *in utero*, it was not possible to examine the effect of COUP-TFII knockouts on Na^+/H^+ exchanger expression. Nevertheless these studies demonstrate the importance of a transgenic approach to study regulation of the Na^+/H^+ exchanger. The results obtained with intact animals help to clarify the roles of different isoforms of transcription factors that were shown to be important in intact cells.

3. TRANSGENIC STUDIES ON THE NHE2 AND NHE3 ISOFORMS OF THE Na^+/H^+ EXCHANGER

3.1 Gastrointestinal Function

Animals exhibiting targeted disruptions against NHE2 and NHE3 have also been utilized to explore the role of these isoforms in both physiology and pathophysiology. Although multiple NHE isoforms are expressed in the gastrointestinal tract, it appears that it is NHE2 deficiency that results in major gastrointestinal defects. NHE2 is present in a number of cell types including mucous, zymogenic and parietal cell (34) and it has been suggested that NHE2 deficient mice may represent an excellent experimental model for achlohydra (absence of hydrochloric acid production in the

stomach) (35). Mice lacking the NHE2 gene develop gastritis early in life concomitant with a large reduction in parietal cells suggesting that NHE2 is particularly of importance in ensuring long term parietal cell viability (34). With increasing age, this form of gastritis is characterized by lymphocyte infiltration as well as marked hyperplasia of various cell types (35).

While NHE2 is also present on intestinal brush border membranes, NHE2 knockout mice do not demonstrate any changes in terms of intestinal Na^+ transport (36, 37). In contrast, NHE3 appears to be the primary isoform responsible for Na^+ transport, at least in the murine intestinal tract. NHE3 knockout mice exhibit marked inhibition of Na^+ absorption resulting in congenital diarrhea (36,37). This is partially compensated for by an amiloride-sensitive electroneutral process (37). Furthermore, recent evidence suggests that upregulation of interferon-γ produced by lymphoid tissue in the gastrointestinal tract is a further compensatory mechanisms which increases gastrointestinal fluidity under NHE3 deficiency (38).

3.2 Renal Tubular Function

NHE3 represents one of the major renal tubular Na^+ transporters especially in the proximal convoluted tubule as well as the thick ascending limb and as such targeted disruption of the NHE3 would be expected to produce marked phenotypic changes in renal function and sodium homeostasis. Mice with NHE3 null mutations maintained on a Na^+ restricted diet exhibited numerous manifestations of disturbed electrolyte and fluid balance including hyperkalemia, urinary salt wasting, hypotension and reduced glomerular filtration rate (39-42). Interestingly, these animals appear to have a function more normal than would be expected which has been attributed to compensatory mechanisms that have not been clearly defined (40-42). For example, the decreased Na^+ absorption in NHE3 knockouts is much less than what should occur in view of the important role that NHE3 plays in this process. Some of the compensatory mechanisms suggested for this include diminished glomerular filtration rates, increased levels of the sodium-phosphate cotransporter in the proximal tubule, as well as an upregulation of 70-kDa form of the epithelial sodium channel (43).

Thus, the overall consequences of NHE2 and NHE3 deficiency in knockout mice, but particularly for NHE3, are defective renal and gastrointestinal functions. NHE3 deficiency in particular appears to have profound and diverse effects which are countered to a marked degree by various compensatory mechanisms which limit the deleterious consequences.

4. PITFALLS IN TRANSGENIC STUDIES

While studies using transgenic approaches to examine Na^+/H^+ exchanger function have yielded a surprising amount of novel and interesting results, there are potential pitfalls that can occur when using transgenic animal models. For example, there have been various documented cases whereby knockout or inactivation of one member of a gene family has resulted in compensatory increase in other members of a gene family (44,45). In this regard it has been demonstrated that in the case of NHE3 knockout mice, there is a compensatory increase in the activity and mRNA levels of the anion exchanger (AE-1). A H^+-K^+-ATPase and a H^+-ATPase were also increased in activity in some renal tissues and contributed to compensatory HCO_3^- reabsorption. It was suggested that upregulation of the proteins could be due to either a metabolic acidosis induced by NHE3 knockout or HCO_3^- excess due to a defect induced by the lack of NHE3 (46). Thus it is clear that at least in some cases, lack of a Na^+/H^+ exchanger can cause an upregulation in the level of another pH regulatory protein. For the NHE1 knockout it has not been documented whether there are changes in the level of other pH regulatory protein such as the anion exchangers. Such a determination would be most useful. It was surprising that the NHE1 knockout mouse was able to survive and in some cases reach maturity considering the many different physiological functions that the protein is involved in (reviewed in (9)). This compensation by other members of a family may be of particular concern where there are several closely related proteins that overlap in function. This could be significant in cases such as with COUP-TF or AP-2 transcription factors that regulate transcription of NHE1 (26).

In experiments whereby genes are inserted into mice at random chromosomal sites, it may sometimes be necessary to consider that the location of the transgene could affect the resultant function. For example in the case of NHE1 overexpressing mice, the human elongation factor 1α promoter was used. It was expected that tissues would universally express the Na^+/H^+ exchanger however in contrast, expression was not universal and varied among tissues (47). The regulation of a transgene may be altered by the site of the chromosomal integration or by the copy number (48,49). This may explain the lack of expression of the NHE1 message in some tissues.

Several approaches can be used to overcome these difficulties. To examine the tissue specific regulation of the NHE1 promoter we made independent mouse transgenic lines. The same results were found with different transgenic lines making it highly unlikely that the results were due to effects of surrounding genomic DNA on a particular insertion site (25). Similarly, another independently made cell line with a different reporter (ß-

galactosidase) gave the same results as with green fluorescent protein suggesting that the results shown were not due to the reporter itself (26). For knockout mice or with insertion of transgenes, it is often difficult to differentiate between developmental and physiological effects that occur as compensatory changes. Though difficult experimentally, the use of controlled transgenes with time dependent, tissue specific or inducible expression can alleviate these problems (49).

5. CONCLUSION

Transgenic animal models are a powerful system for examination of biological questions. Using transgenic approaches it has been possible to examine the physiological function of the NHE1, NHE2 and NHE3 isoforms of the Na^+/H^+ exchanger. In several cases the experiments have yielded unexpected results. For example, for NHE1, the discovery of a slow wave epilepsy associated with absence of the protein was not predicted. The availability of various Na^+/H^+ exchanger gene knockouts has allowed the comparative effects of the knockouts to be examined in several cells lines. This is a unique approach to the study of physiological function. While the transgenic animal models are a powerful tool for the study of the Na^+/H^+ exchanger family, the approach is not without drawbacks. Caution must be used to ensure that in the intact animal, compensatory effects are not masking true physiological functions. In addition, when genes are inserted randomly into chromosomal sites, it is necessary to consider that the location and number of the transgenes could affect biological functions.

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Chapter 8

pH-REGULATORY MECHANISMS IN THE MAMMALIAN OOCYTE AND EARLY EMBRYO

Jay M. Baltz
Ottawa Health Research Institute and University of Ottawa, Ottawa, Ontario, Canada

1. INTRODUCTION

Na^+/H^+ exchangers and intracellular pH (pH_i) regulation assumed a central position in the study of fertilization following the discovery that egg activation in the sea urchin is triggered in part by a substantial pH_i increase immediately after fertilization, and that this increase is mediated by the activation of Na^+/H^+ exchange (1). In mammals, the regulation of pH_i has been extensively studied in early embryos, with more recent attention given to the role of pH_i-regulatory mechanisms during fertilization and meiosis in the oocyte. This review will focus principally on pH_i-regulatory mechanisms in mammalian oocytes and preimplantation embryos during meiosis, fertilization, and embryo development.

2. OOCYTE AND EMBRYO DEVELOPMENT

Oocytes are naturally arrested in prophase of first meiosis throughout their growth in the ovary (2). Fully grown, prophase I-arrested oocytes are called "germinal vesicle" (GV) stage oocytes, referring to the characteristic prominent nucleus with a single, large nucleolus (Fig. 1). In most mammals, the oocyte remains arrested in prophase I until ovulation, when the oocyte is released from prophase arrest and enters first meiotic metaphase (MI). MI culminates in an unequal cytokinesis, with the first polar body carrying away one-half of the genetic material, whereupon the oocyte immediately enters second meiotic metaphase (MII) where it is again arrested until fertilization (3,4).

Following fertilization, the oocyte is released from MII arrest, with cytokinesis occurring several hours later to produce a second polar body and a haploid egg. The male and female haploid genetic material then form separate "pronuclei" (Fig. 1),

which persist in mammals until the end of the fertilized egg or 1-cell embryo stage, with the maternal and paternal genetic material first combining in most mammals during first mitosis

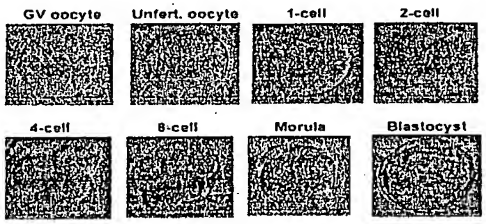

Figure 1. Mammalian oocyte and preimplantation (PI) embryo development. Micrographs of mouse oocytes and PI embryos, showing the germinal vesicle (GV), zona pellucida (ZP), pronuclei (PN), polar bodies (PB), inner cell mass (ICM), trophectoderm (T) and blastocoel cavity (B). All are shown at same scale; oocytes are ~ 80 μM in diameter.

The embryo then undergoes a series of reductive mitotic cleavages in which the volume of the cells is reduced with each successive generation (Fig. 1). This occurs for several cell cycles, continuing in the mouse, for example, until the 8- to 16-cell stage. Embryos from this period are collectively known as cleavage-stage embryos. The embryo then expresses cell adhesion proteins and forms gap junctions, so that the previously independent cells form the first integrated tissue of the developing animal, the morula. The outer cells of the morulae begin to differentiate into a fluid-transporting epithelium, and a fluid-filled cavity forms and expands to comprise most of the embryo. This is then termed a blastocyst (5). The epithelium—the trophectoderm—forms a thin, spherical layer surrounding the blastocoel cavity. A group of cells, called the inner cell mass (ICM), adheres to the interior of the trophectoderm within the blastocoel. The ICM is fated to form the fetus, while extraembryonic tissues arise from the trophectoderm. Embryos from the fertilized egg through blastocyst are collectively designated preimplantation (PI) embryos in mammals. At the end of the PI period, the fully-expanded blastocyst implants in the uterine wall.

3. EXPRESSION OF Na^+/H^+ EXCHANGER (NHE) AND ANION EXCHANGER (AE) ISOFORMS IN MAMMALIAN PI EMBRYOS

NHE-1 mRNA has been shown to be present in unfertilized eggs and at all PI stages, including cleavage-stage embryos, morulae, and blastocysts (6). NHE-1 protein becomes localized to the inner, or basolateral, surface of the trophectoderm and the ICM at the blastocyst stage (6). In contrast, NHE-3 mRNA was found in the unfertilized egg but not in the blastocyst of the mouse. Interestingly, however, NHE-3 protein persists in the blastocyst, becoming localized to the outer (apical) surface of the trophectoderm (6). This is consistent with previous work which indicated that fluid transport into the mouse blastocoel requires Na^+ transport across the apical trophectoderm, possibly via a Na^+/H^+ exchanger (7). NHE-2 mRNA was not detected in mouse eggs or embryos (6). NHE-4 mRNA was not present in PI mouse embryos (6,8), but has been found in unfertilized eggs (8). Expression of other NHE isoforms has not been assessed.

AE family HCO_3^-/Cl^- (anion) exchangers are also expressed in PI mouse embryos. mRNA for AE-2 was detected at all stages of PI embryos (9) and in unfertilized eggs (10). In contrast, AE-3 mRNA was first detected in 2-cell embryos, persisting through the blastocyst stage (9). AE-1 was not detected at any stage (9). AE-2 protein was evident in the plasma membranes of cleavage-stage mouse embryos, and becomes localized to the inner, basolateral surface of the trophectoderm where it may have a role in Cl^- transport across the trophectoderm (11).

4. ACTIVITY OF pH_i-REGULATORY MECHANISMS IN PI EMBRYOS

While the external pH optimum for in vitro mouse embryo development had been first determined in the 1960's (12), it is only relatively recently that the mechanisms by which embryos regulate pH_i have begun to be investigated. The most extensive study has been of mouse and hamster PI embryos, with some work also reported in the cow and human.

4.1 Na^+/H^+ Exchanger Activity And Recovery From Acidosis In PI Embryos

The first work done to elucidate pH_i regulation in PI embryos was in mouse 2-cell embryos. In these studies, baseline pH_i was found to be low—below pH 7.0—and no NHE-mediated recovery from experimentally-induced acidosis was evident. Recoveries occurred, but were unaffected by absence of Na^+ or by amiloride or its derivatives, implying an absence of Na^+/H^+ exchanger activity (13). Similar investigations also failed to reveal any HCO_3^--dependent recovery from acidosis (13).

Several years later, a different result was reported, where robust recovery from acidosis was demonstrated in mouse 2-cell embryos (15). Here, recovery was Na^+-dependent and inhibited by amiloride derivatives, indicating that Na^+/H^+ exchanger activity regulated pH_i in these embryos. The set-point below which the exchanger was activated was about 7.4.

Hamster embryos were also found to exhibit robust Na^+/H^+ exchanger activity (16). One-cell through 8-cell stage hamster embryos all possessed active Na^+/H^+ exchange which regulated pH_i. The exchanger in hamster was activated below a set-point of 7.14. Recovery from acidosis in hamster embryos was completely Na^+-dependent with no dependence on HCO_3^- (16), indicating that the Na^+/H^+ exchanger

is the sole mechanism mediating recovery from acidosis in hamster cleavage-stage embryos. Embryo development was impaired when Na^+/H^+ exchanger activity was inhibited under conditions which favored intracellular acidosis (16), indicating a physiological requirement for Na^+/H^+ exchanger activity and NHE expression in hamster PI embryos. Similarly, bovine embryos regulated pH_i via Na^+/H^+ exchange, with exchanger activity evident at the 2-cell, 4-cell, and 8-cell stages (17).

These findings prompted a re-examination of 2-cell mouse embryos. Different strains of mice had been used, with CF1-derived embryos apparently lacking Na^+/H^+ exchanger activity, while activity was found in embryos of the QS strain. Thus, it was possible that strain differences existed in pH_i regulation in mouse embryos and was at least partly responsible for the divergent results.

When 2-cell embryos derived from females of three mouse strains—CF1, BDF, and Balb/c—were tested (QS were unavailable), Na^+/H^+ exchanger activity (Na^+-dependent, amiloride-sensitive recovery) was detectable in each (18), including low but detectable activity in CF1 embryos, contrary to previous results. The set-point below which the Na^+/H^+ exchanger was activated was similar in each strain, and fell between 7.15-7.20 (18). Activity was, however, very low compared to hamster (Fig. 7-2) and at least 5-fold lower than that of QS embryos (18). The reasons for the apparent strain-dependent differences in pH_i regulation in mouse embryos remain obscure, but further discussion can be found in the original reference (18).

Another strain difference in 2-cell mouse embryo Na^+/H^+ exchanger activity was found in experiments in which cytoplasmic acidification was induced in Na^+-free medium, and then Na^+ replaced after a period of 10 minutes (18). The recoveries from acidosis that occurred upon Na^+ reintroduction in CF1 and BDF embryos were not significantly different than those in which there was no period in Na^+-free medium. However, in Balb/c strain embryos, recovery was stimulated approximately 5-fold when they spent a period in Na^+-free medium before recovery was induced by reintroduction of Na^+ (18). Other experiments indicated that this

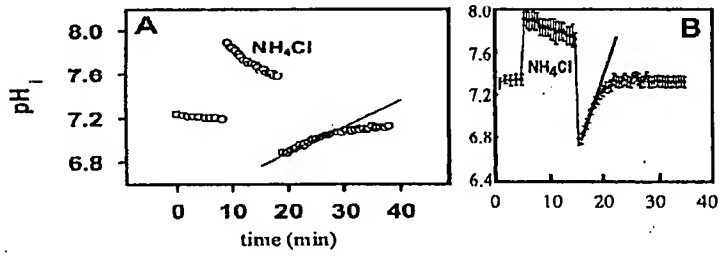

Figure 2. Recoveries from acid loads induce by NH_4Cl pulse in CF1 mouse (A) and hamster (B) 2-cell embryos. Panels have been adjusted so that the scales are approximately equal, to show the relatively slow recovery by mouse embryos (initial rate of recovery indicated by slopes of lines). Adapted from Steeves et al. (18) (A) and Lane et al. (30) (B) with permission of the Society for the Study of Reproduction.

was not simply due to intracellular Na^+ depletion and hence steepening of the Na^+ gradient favoring H^+ transport, but rather was due to the absence of Na^+ itself during acidosis (18).

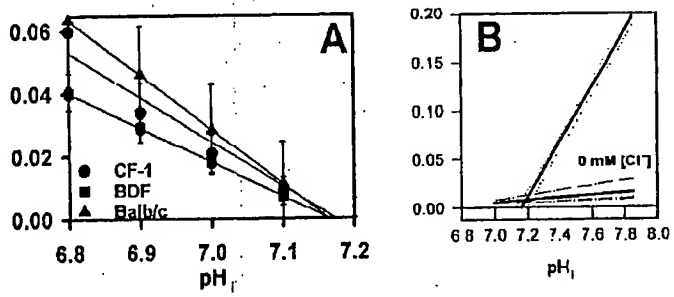

Figure 3. Activity of HCO_3^-/Cl^- exchanger in mouse embryos at 1-cell, 2-cell, morula, and blastocyst stages. Gray bars indicate activity measured as described in Zhao et al., (9), while open bars indicate residual, non-specific activity when the HCO_3^-/Cl^- exchanger is inhibited by DIDS. Adapted from Zhao et al., (9) with permission of the American Society for Biochemistry and Molecular Biology.

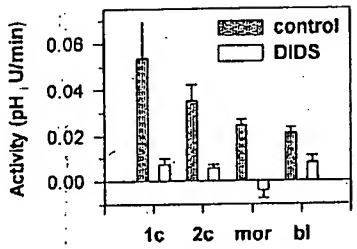

Figure 4. Set-points of Na^+/H^+ (A) and HCO_3^-/Cl^- (B) exchangers in 2-cell mouse embryos (strains indicated in A, CF1 strain in B). Activity was measured as initial rate of recovery from induced acidosis for Na^+/H^+ exchange (A) or induced alkalosis for HCO_3^-/Cl^- exchange (B), in pH units/min as a function of pH_i. In B, 0 mM $[Cl^-]$ indicates non-specific recovery in the absence of external Cl^-. Adapted from Steeves et al., (18) (A) with permission of the Society for the Study of Reproduction and from Zhao and Baltz, (21) (B) with permission of the American Physiological Society.

Most recently, the pharmacological properties of recovery from an induced acid load have been used to deduce the relative contributions of NHE isoforms NHE-1, -2, and -4 to pH_i regulation in QS mouse embryos (8). pH_i regulation by NHE-1 appeared to persist through all of PI development and into the blastocyst stage, where activity was localized to the ICM but not found in the trophectoderm, while an additional component of pH_i regulation attributable to NHE-3 was present only in earlier stages, decreasing by the 2-cell stage and lost by the blastocyst stage (8).

NHE-3 protein, however, is still found in trophectoderm (above; 6), implying that NHE-3 may be inactive in pH_i regulation but function in Na^+ and fluid transport in the blastocyst. Activity attributed to NHE-4 was also found in blastocysts, in the ICM only (8). Since mRNA for NHE4 was only found in the oocyte, this would imply that the protein is stored for several days before becoming functional in the blastocyst (8).

Human embryos presented a complex picture of pH_i regulation in the acid range. When cleavage stage human embryos were placed in external media (HCO_3^--free) which was slightly more acidic than the usual medium (pH 7.0 vs. 7.5), their cytoplasm acidified to nearly the external level and did not recover (19). This was true for GV and MII oocytes as well as all cleavage stages and morulae. Blastocysts, in contrast, fully recovered their original baseline pH_i.

Subsequently, pH_i regulation was examined in human cleavage-stage embryos subjected to more profound acidosis (to about pH_i 6.6) induced by NH_4Cl pulse (10). This revealed Na^+/H^+ exchange that was activated below a pH_i of about 6.8 and mediated recovery from acidosis to about this level (10). In addition, when HCO_3^- was present, a second component of recovery appeared that allowed further recovery up to about pH_i 7.1. Thus, human cleavage-stage embryos appear to have at least two mechanisms for pH_i regulation in the acid range, an NHE-type Na^+/H^+ exchanger, and a second, HCO_3^--dependent mechanism.

4.2 HCO_3^-/Cl^- Exchanger Activity And Recovery From Alkalosis In PI Embryos

pH_i regulation in the alkaline range was first studied in mouse 2-cell embryos, where it was shown that recovery from alkalosis required external Cl^- and HCO_3^-/CO_2, and was inhibited by the presence of the anion exchange inhibitor, DIDS (20). In addition, a large component of Cl^- transport in 2-cell mouse embryos was HCO_3^--dependent, again indicating significant HCO_3^-/Cl^- exchanger activity (20). pH_i regulation by this mechanism is also important to embryo development, as its inhibition was highly detrimental to embryo development in vitro under conditions favoring intracellular alkalosis (9).

Mouse PI embryos are able to recover from intracellular alkalosis via HCO_3^-/Cl^- exchanger activity at all stages examined (21). At each stage, the set-point above which the exchanger is activated is within the range of 7.1-7.2, and does not change much with development (21). Hamster embryos also regulate pH_i via HCO_3^-/Cl^- exchanger activity, with a set-point of about 7.2 (17). Human cleavage-stage embryos also possess HCO_3^-/Cl^- exchanger activity at all stages examined (10,19), with a set-point of 7.2 -7.3 (10).

In mouse, HCO_3^-/Cl^- exchanger activity is highest in 1- and 2-cell embryos (Fig. 3), falling off by the morula and blastocyst stages (9,21); this may reflect the environment of the embryo, since oviductal fluid, in which cleavage-stage embryos reside, is thought to be significantly more alkaline than the uterine fluid surrounding blastocysts. At the blastocyst stage, about 80% of the HCO_3^-/Cl^- exchanger activity is apparently located on the inner trophectoderm surface or ICM (11), perhaps reflecting localization of AE2 protein (above).

Thus, the HCO_3^-/Cl^- exchanger and Na^+/H^+ exchanger activities in PI embryos together maintain pH_i within a narrow range around 7.2, since both mechanisms have set-points in embryos near this value (Fig. 4).

5. pH_i REGULATORY MECHANISMS DURING MEIOSIS AND FERTILIZATION

Control over pH_i occupies a key role in the physiology of fertilization, since an increase in pH_i is a required part of egg activation in what is perhaps the best-studied model of fertilization—the sea urchin. Although the physiological role of the pH_i increase at fertilization in the sea urchin, and the signaling pathways which control this activation, have been elucidated in detail, any role for pH_i or pH_i-regulatory mechanisms in mammals remained unexploreduntil very recently.

5.1 Changes In pH_i At Fertilization

A role for pH_i in the activation of eggs at fertilization had been suspected since the early part of the last century, when it was found that sea urchin eggs could be parthenogenetically activated by weak, cell-permeant bases, and that fertilized eggs released acid. Together, these observations implied that egg activation involved alkalinization of the cytoplasm (22). However, whether pH_i increased at fertilization or whether the "fertilization acid" was generated by other means was the subject of considerable debate for decades, with a pH_i increase at fertilization not conclusively demonstrated until the 1970s when direct measurements showed an approximately 0.3-0.5 pH unit increase in pH_i occurring within minutes of sperm penetration of the egg (1,23). This increase in pH_i is sufficient to trigger most of the events of egg activation which follow normal fertilization, and is necessary for the onset of embryo development (22).

A breakthrough in elucidating the mechanism of the pH_i increase in sea urchin eggs at fertilization came about when Epel's laboratory first demonstrated dependence upon external Na^+, a 1:1 stoichiometry of Na^+ influx and H^+ efflux, and amiloride inhibition (1), providing one of the first clear demonstrations of Na^+/H^+ exchange in any system. Subsequent investigations established a key role for the exchanger in fertilization, and elucidated the signaling pathways which control its activation (reviewed in 22).

While a number of other invertebrates were shown to exhibit a similar pH_i increase at fertilization, this did not prove to be universal. For example, starfish eggs were found to exhibit a high pH_i even before fertilization which did not change significantly after fertilization (23).

Whether pH_i increased at fertilization in mammalian eggs was not resolved until fairly recently. Several reports showed that there was no appreciable alteration in pH_i during the first few hours after fertilization or parthenogenetic activation in mouse or rat (25-27), ruling out a role for a pH_i increase in initiating embryo development. In addition, resting pH_i in unfertilized eggs is similar to that of 1-cell embryos approximately 12 hours post-fertilization (within about 0.1 pH units), ruling out any major change in pH_i after mammalian egg activation (8,27).

5.2 pH_i Regulation In The Egg At Fertilization In The Mammal

Since there was no change in pH_i at fertilization, it was expected that unfertilized mammalian eggs would exhibit the same levels of activity of pH_i-regulatory mechanisms such as Na^+/H^+ exchanger and HCO_3^-/Cl^- exchanger as had been found in fertilized eggs and embryos. Thus, it was surprising when it was found that these activities were nearly undetectable in unfertilized mouse or hamster eggs. Instead, the Na^+/H^+ exchanger in hamster (28) and HCO_3^-/Cl^- exchanger in mouse (CF1 strain) and hamster (17,29) were nearly quiescent in MII eggs, and unfertilized eggs were not able to recover from acid or alkaline loads by the mechanisms which had been shown to be present and highly active in 1-cell embryos (Fig. 5). At least some Na^+/H^+ antiporter activity has been reported in the unfertilized mouse (QS strain) egg, although the activity relative to the 1-cell embryo was not specified (8). In addition, human unfertilized eggs may exhibit a small amount of Na^+/H^+ exchanger activity regulating pH_i, but the activity appears low compared to that in embryos (10).

Na^+/H^+ exchange activity in the hamster and HCO_3^-/Cl^- exchange activity in the mouse were each found to develop slowly over the course of several hours after sperm-egg fusion (Fig. 6), with full activity being reached only around the time pronuclei are formed (22,29) as the activated egg exits metaphase. Upregulation of both exchangers was independent of protein synthesis and did not require an intact cytoskeleton or Golgi, implying that their appearance was due to activation of pre-existing exchangers rather than de novo synthesis of NHE or AE proteins (22,29). Thus, although pH_i does not change as a consequence of fertilization in mouse and hamster eggs, they nonetheless resemble those of sea urchin, with fertilization and egg activation leading to the activation of pH_i-regulatory mechanisms.

5.3 pH_i Regulation During Meiosis

The low Na^+/H^+ or HCO_3^-/Cl^- exchanger activity in unfertilized mouse or hamster eggs naturally raised the question of whether their oocytes were similarly incapable of regulating pH_i before meiotic maturation, or whether these mechanisms became inactivated during meiosis. The fully grown oocyte is arrested in the ovarian follicle as a GV oocyte and then is induced to proceed through meiosis upon ovulation (see below). Activity of HCO_3^-/Cl^- exchanger was assessed during meiosis in the mouse, with this pH_i-regulatory mechanism chosen for study due to the low and variable activity of Na^+/H^+ exchange in available mouse embryos.

Unlike MII eggs, GV oocytes possessed essentially the same high HCO_3^-/Cl^- exchanger activity as 1-cell embryos (31). When oocytes were meiotically matured in vitro, exchanger activity gradually decreased during first meiotic metaphase (MI), until HCO_3^-/Cl^- exchanger activity became nearly undetectable just before the transition to MII (Fig. 7). In contrast, oocytes maintained in GV arrest in vitro (using the cell-permeant cAMP analog, dibutyryl cAMP to mimic natural GV arrest), HCO_3^-/Cl^- exchanger activity was maintained (31).

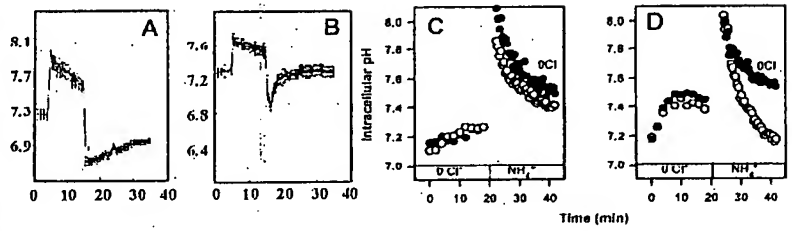

Figure 5. Na^+/H^+ and HCO_3^-/Cl^- exchanger activities in unfertilized eggs vs. 1-cell embryos. A,B. Recovery from NH_4Cl pulse-induced acid load in hamster MII eggs (A) and 1-cell embryos (B). C,D. Recovery from an induced alkalosis in mouse CF1 strain eggs (C) and 1-cell embryos (D). Here, eggs or embryos were exposed to Cl^--free medium (t=0), which causes any HCO_3^-/Cl^- exchanger to run in reverse and results in intracellular alkalinization (seen in D). Then, substantial intracellular alkalinization was induced with NH_4Cl, and specific recovery via HCO_3^-/Cl^- exchange revealed as the component which requires external Cl^-. Adapted from Lane et al. (28) (A) and Phillips and Baltz, (29) (C,D) with permission of Elsevier Science (USA) and Lane et al. (30) (B) with permission from the Society for the Study of Reproduction.

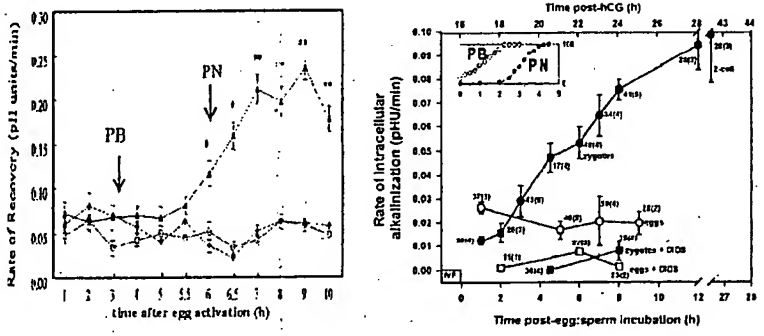

Figure 6. Activation of Na^+/H^+ exchanger in hamster eggs (left) and HCO_3^-/Cl^- exchanger in mouse (CF1 strain) eggs (right) following egg activation. Eggs were fertilized at t=0, and polar bodies (PB) and pronuclei (PN) developed at the average times indicated. Na^+/H^+ exchanger activity (left) is shown by triangles, while curves at bottom show nonspecific activity in the presence of an amiloride derivative (circles) or absence of Na^+ (crosses). HCO_3^-/Cl^- exchanger activity (right) is indicated by closed circles, while activity in eggs maintained under identical conditions is shown as open circles. Nonspecific activity in the presence of DIDS is shown as indicated. Adapted from Lane et al., (22) (left) and Phillips and Baltz, (29) (right) with permission from Elsevier Science (USA).

This inactivation is specific to meiotic metaphase, rather than being a general feature of any metaphase, at least in eggs and early embryos. In contrast to metaphase-arrested MII mouse eggs, 1-cell embryos which have entered the first mitotic metaphase at the end of the first cell cycle did not exhibit any decrease in

HCO_3^-/Cl^- exchanger activity (31). Even when these embryos were experimentally arrested in first mitotic metaphase (by pharmacologically disrupting the mitotic spindle), HCO_3^-/Cl^- exchanger activity was maintained indefinitely (31).

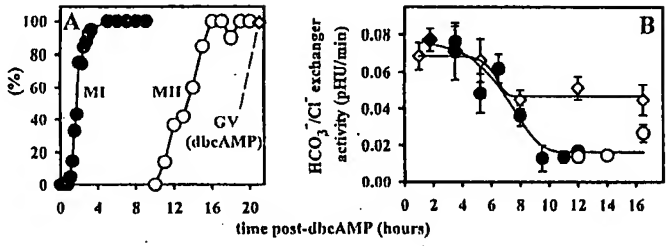

Figure 7. Inactivation of HCO_3^-/Cl^- exchanger during meiosis in mouse oocytes. A. Timecourse (% of oocytes) of entry into MI and MII following removal of oocytes from dibutyryl cAMP (no oocytes entered meiosis in the presence of dbcAMP, as indicated by open diamond). B. HCO_3^-/Cl^- exchanger activity in oocytes during meiosis. Filled diamond indicates GV oocytes, filled circles indicate MI oocytes, while open circles indicate MII. Activity in GV oocytes maintained with dbcAMP is shown by open diamonds. Adapted from Phillips et al., (31) with permission from the American Society for Cell Biology.

Thus, mammalian oocytes in the ovary appear to be capable of regulating pH_i similarly to embryos. However, during progression through meiotic metaphase, the HCO_3^-/Cl^- exchanger becomes gradually inactivated, so that activity is quiescent in mature MII eggs awaiting fertilization. The physiological reason for this specific meiotic inactivation remains unclear, although it may be related to the need to conserve energy or maintain transmembrane ionic gradients in the ovulated egg until fertilization and metabolic activation.

5.4 Regulation Of HCO_3^-/Cl^- Exchanger Activity During Meiosis

Release of the GV oocyte from prophase I arrest is initiated by the activation of the universal M phase promoting factor (MPF), consisting of activated cdk1 kinase complexed with cyclin B (32,33). Meiotic metaphase, however, is characterized by an additional activity—cytostatic factor (CSF)—that stabilizes MPF and induces MII arrest (34). CSF activity is due, at least in part, to activation of the MOS-MEK-MAP kinase (MAPK)-p90 RSK pathway in the egg during MI. While MPF is common to both meiotic and mitotic metaphase, CSF is unique to meiotic metaphase. Since MPF and CSF are active only during meiotic metaphase in the oocyte—when HCO_3^-/Cl^- exchange is downregulated—they were possible candidates for the regulatory mechanism mediating its inactivation.

Measurements of MAPK and MPF activity showed that HCO_3^-/Cl^- exchanger activity was inversely correlated with both during meiosis. GV oocytes had high HCO_3^-/Cl^- exchanger activity and no detectable MAPK or MPF activity, while MI

oocytes just prior to the MI/MII transition had developed high MAPK and MPF activity while HCO_3^-/Cl^- exchanger activity has reached its minimum (31). The re-appearance of HCO_3^-/Cl^- exchanger after egg activation, however, occurred much later than inactivation of MPF, but nearly coincident with CSF inactivation (31), following essentially the same timecourse (Fig. 8). Thus, it appeared that CSF and the MAPK pathway exhibited the best temporal correlation with inactivation of HCO_3^-/Cl^- exchange.

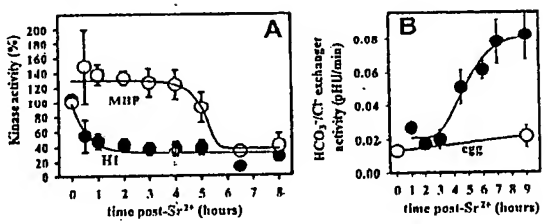

Figure 8. Reactivation of HCO_3^-/Cl^- exchanger after egg activation in the mouse. A. Inactivation of MPF (measured as histone H1 kinase, H1) and MAPK (measured as myelin basic protein kinase, MBP) after egg activation (parthenogenetically activated using Sr^{2+}). B. Timecourse of reactivation of HCO_3^-/Cl^- exchanger. Adapted from Phillips et al., (31) with permission from the American Society for Cell Biology.

MPF and CSF activity in mouse oocytes are usually coupled, since CSF stabilizes MPF and MPF supports CSF activity. Thus, although MPF activation induces release of GV oocytes from prophase I arrest before MAPK activity is detectable, MAPK activation follows soon after. Similarly, egg activation is accompanied by loss of MPF activity, but MAPK deactivation and the loss of CSF activity in the egg soon follows. It is possible, however, to pharmacologically decouple MPF and CSF activities in oocytes and eggs, and this could be used to investigate possible regulation of HCO_3^-/Cl^- exchanger activity in oocytes.

In activated or fertilized eggs, high MAPK activity can be indefinitely maintained by the phosphatase inhibitor okadaic acid while MPF activity undergoes its normal decrease after egg activation (35). Activated mouse eggs did not develop HCO_3^-/Cl^- exchanger activity when treated with okadaic acid, consistent with negative regulation by MAPK but not MPF (31). In contrast, the MAP kinase pathway can be inactivated in unfertilized MII eggs using U0126, a specific inhibitor of the MAPK kinase, MEK (36). When MAPK was thus inhibited in MII eggs, HCO_3^-/Cl^- exchanger activity was found to develop precociously in the resulting parthenogenetically-activated eggs (31), indicating that inactivation of MAPK may permit activation of the HCO_3^-/Cl^- exchanger in eggs. Similar findings were obtained in oocytes during meiosis, where experimentally activating MAPK in GV oocytes in the absence of MPF activation caused a loss of HCO_3^-/Cl^- exchanger activity. Conversely, oocytes which progressed through MI and developed normal high MPF activity would retain HCO_3^-/Cl^- exchanger activity if MAPK activity was suppressed (31). Together, these implied that HCO_3^-/Cl^- exchange is inhibited in oocytes by the CSF pathway in mouse oocytes during meiosis.

Thus, our current understanding is that the HCO_3^-/Cl^- exchanger in oocytes is inactivated during the course of first meiotic metaphase due to the development of CSF activity and activation of the MAPK pathway. The high MAPK activity in MII eggs suppresses HCO_3^-/Cl^- exchanger activity until after fertilization. As MAPK activity disappears, coincident with exit from metaphase and the development of pronuclei, HCO_3^-/Cl^- exchanger activity reappears. Since Na^+/H^+ exchanger activity follows a similar pattern after fertilization in hamster, it may be similarly regulated, although this remains to be tested, as does the status of Na^+/H^+ exchanger activity in eggs of mouse and other mammalian species.

6. CONCLUSION

pH_i regulation is active from the earliest stages of development in mammals, and both NHE family Na^+/H^+ exchangers and AE family HCO_3^-/Cl^- exchangers are expressed and regulate pH_i from the 1-cell embryo stage. It has recently become clear that regulation of both types of exchangers in oocytes and eggs is complex. Our current understanding is that at least some pH_i-regulatory mechanisms are largely inactive in meiosis in mammals, and reactivated only several hours after fertilization, probably under the control of MAP kinase. Further work is needed to elucidate meiotic regulation of NHE and AE exchangers in oocytes, and to investigate the physiological reasons for inactivation of pH_i regulation in meiosis.

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Chapter 9

Na^+/H^+ EXCHANGER ACTIVATION BY MYOCARDIAL STRETCH

An Autocrine/Paracrine Loop

Horacio E. Cingolani, Néstor G. Pérez, Irene L. Ennis and María C. Camilión de Hurtado

Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina

1. INTRODUCTION

The sarcolemmal Na^+/H^+ exchanger (NHE) extrudes intracellular H^+ in exchange for extracellular Na^+ and has a role in the regulation of not only intracellular pH (pH_i) but also intracellular Na^+ (Na^+_i) homeostasis. Both H^+ and Na^+ are important determinants of cardiac contractility and therefore, the level of NHE activity may become relevant to many (patho)physiological conditions. We have examined the participation of NHE activity in the response to myocardial stretch. Pioneering studies performed by several independent research groups on neonatal rat cardiomyocytes cultured on deformable silicone dishes showed that mechanical stress induces a whole set of cell responses including the expression of immediate-early and fetal genes, secretion of growth factors such as angiotensin II (Ang II) and endothelin-1 (ET-1), activation of PKC and MAPK-dependent intracellular signaling pathways, and increased protein synthesis (1-4). Since protein synthesis and MAPK activation were partially abrogated by inhibitors of NHE activity (4), the possibility of stretch-induced NHE stimulation was suspected. Our experiments were designed to assess, in papillary muscles of adult animals, the mechanism(s) of NHE activation by myocardial stretch and its contribution to the subsequent increase in force. The experimental set-up is schematically illustrated in Figure 1. Our data support the theory that the stretch of cardiac muscle elicits an autocrine/paracrine chain of events involving Ang II-ET release and stimulation of NHE activity, which subsequently elevates Na^+_i and promotes the reverse mode of Na^+/Ca^{2+}

exchange (NCX). This in turn increases Ca^{2+}_i transients (CaT) and developed force (DF).

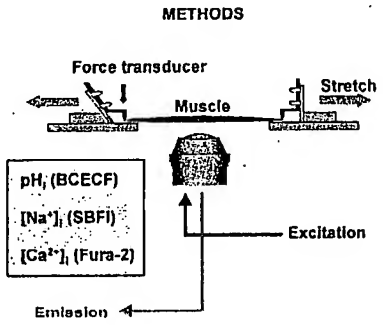

Figure 1. Schematic representation of the set-up. Papillary muscles were mounted between a force transducer and a fixed hook in a perfusion chamber on the stage of an inverted microscope and superfused with either HEPES- or HCO_3^-/CO_2-buffered solutions. Stretch effect on Na^+_i, Ca^{2+}_i and pH_i was monitored by epifluorescence with SBFI and BCECF (AM forms) to measure Na^+_i and pH_i respectively while free Fura-2 was microinjected to determine Ca^{2+}_i transients.

2. MECHANISM OF STRETCH-INDUCED INCREASE OF NHE ACTIVITY.

The results of Figure 2 demonstrate that the stretch of cardiac muscle causes the stimulation of NHE activity through Ang II AT_1 receptors. There is a rise in pH_i after stretch that is cancelled by an inhibitor of NHE activity, ethylisopropylamiloride (EIPA) and by losartan, a highly selective AT_1 antagonist. These results are in accordance with previous ones reported by Sadoshima et al. (1) who demonstrated, by immunoelectron microscopy, that stretch caused the acute release of prestored Ang II from ventricular myocytes, later followed by an increase in angiotensinogen mRNA levels.

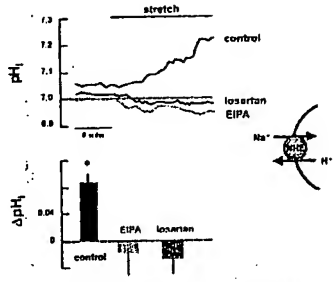

Figure 2. A. Representative records showing the time-course of pH_i changes after stretch in HEPES-buffered superfused muscles, under control conditions and following the inhibition of NHE activity with ethylisopropilamiloride (EIPA) or blockade of Ang II AT_1 receptors with losartan. *B.* Overall results. Modified with permission from Cingolani et al. (5).

Since the antiporter exchanges intracellular H^+ for extracellular Na^+, NHE activity can also be monitored by changes in Na^+_i. Figure 3 shows an increase in Na^+_i after stretch. This effect is cancelled by either EIPA or losartan. These results reinforce the notion that stretch-released Ang II increases NHE activity through the binding to AT_1 receptors.

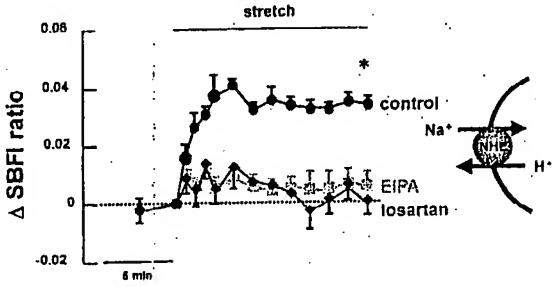

Figure 3. Intracellular Na^+ (assessed by SBFI 340/380 fluorescence ratio) increases after myocardial stretch. The fact that the rise in Na^+ is cancelled both by EIPA and losartan is consistent with the notion that stretch induces the release of prestored Ang II which activates NHE through AT_1 receptors. Modified with permission from Alvarez et al. (8).

The data illustrated in Figures 2 and 3 were obtained in muscles superfused with nominally bicarbonate-free (HEPES-buffered) medium. However, in the presence of HCO_3^-/CO_2 buffer, the activation of NHE by stretch produces an increase of similar magnitude in Na^+_i but not in pH_i (Figure 4). The reason for a lesser pH_i response in this experimental condition is consistent with the finding that under the physiological buffer, Ang II causes together with NHE activation, an increase in acid load carried by the Na^+-independent Cl^-/HCO_3^- exchange (AE).[6,7] Therefore, attention has to be focused on Na^+_i rather than on pH_i in order to detect an increase of NHE activity.

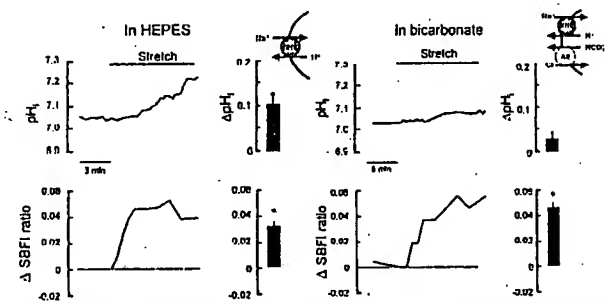

Figure 4. Representative experiments showing that in the presence of the physiological CO_2/HCO_3^--buffer, stimulation of NHE activity by stretch causes the elevation of Na^+_i level (assessed by SBFI 340/380 fluorescence ratio) but not of pH_i, due to the simultaneous activation of Na^+-independent Cl^-/HCO_3^- exchange (AE).

To further characterize the mechanism, the possible involvement of ET-1 in NHE activation by stretch was explored. Compelling evidence demonstrates that many effects previously adscribed to Ang II are actually due to the release/formation of endogenous ET. Figure 5 shows that the increase in Na^+_i elicited by stretch is suppressed by either blockade of ET_A receptors with BQ 123 or inhibition of the endothelin-converting enzyme with phosphoramidon. These results can be interpreted as indicating that stretch-released Ang II increases the conversion rate of bigET to ET, which is, as in many other cases, the final effector of NHE activation.

Whether cardiomyocytes themselves or any other type of intramyocardial cells are the source of ET is unkown at present. In this regard, it should be recalled that Ito et al. showed in cultured isolated myocytes that Ang II causes a PKC-dependent increase in prepro ET-1 mRNA levels and release of ET-1 (9). Further evidence favoring the proposal that myocytes are both the source and the target of ET was recently provided by the experiments of Aiello et al. (10), who measured the outward Na^+/Ca^{2+} exchange current (I_{NCX}) in isolated cat cardiomyocytes. As illustrated in Figure 6, an increase in I_{NCX} was produced by exogenous Ang II and this effect could be abrogated by blocking either Ang II-AT_1 or ET-1 receptors.

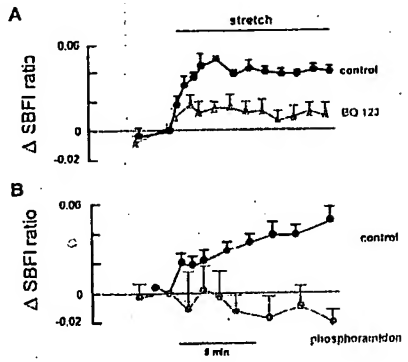

Figure 5. Stretch-induced increases in Na^+_i are cancelled by *A.* selective blockade of ET_A receptors with BQ 123, or *B.* inhibition of the endothelin-converting enzyme by phosphoramidon. These results demonstrate the involvement of endogenous ET in the mechanism leading to NHE activation by stretch.

On the whole, the preceeding data allow to conclude that stretch releases Ang II which in turn induces the release/formation of ET-1 and this peptide then activates NHE. However, a direct Ang II stimulatory effect on NHE cannot completly be ruled out. The fact that blockers of ET receptors suppressed exogenous Ang II- induced NHE activation while losartan failed to inhibit that of ET, strongly argues in favor that the cross-talk between both peptides is in the proposed way and not the opposite.

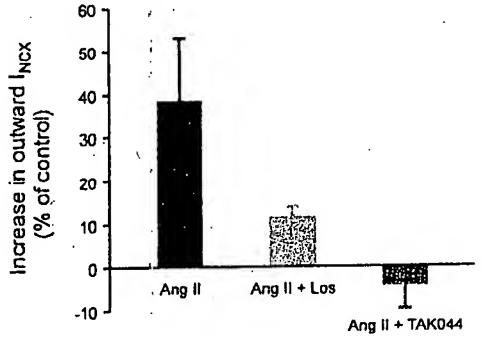

Figure 6. Ang II increases outward I_{NCX} through AT_1 receptors in isolated myocytes. This effect is cancelled after the blockade of ET-1 receptors with TAK044 indicating that stimulatory effect of exogenous Ang II is mediated by endogenous ET-1. Modified from Aiello et al. (10).

3. MECHANICAL COUNTERPART OF STRETCH-INDUCED INCREASE OF NHE ACTIVITY

Since the increase in Na^+_i induced by stretch should change the thermodynamic balance of NCX favoring an increase in intracellular Ca^{2+}, the effect of stretch on CaT was explored. As suspected, the rise in Na^+_i is accompanied by a slow increase in CaT. Figure 7 shows that, while there is no change immediately after stretch, peak systolic Ca^{2+} and CaT are significantly increased after 15 min (~20% increase in CaT).

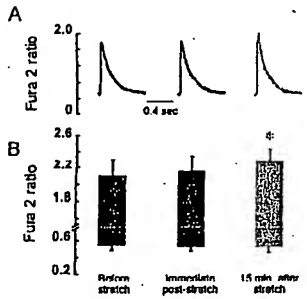

Figure 7. Time-course of intracellular Ca^{2+} changes after stretch. *A.* Representative CaT (assessed by Fura-2 ratio) recorded at 3 different time-points of the stretch protocol. *B.* Averaged (±SEM) values of peak systolic (upper floating columns) and diastolic (bottom floating columns) ratios. (*) indicates significant difference compared to values before stretch. Modified with permission from Alvarez et al. (8).

Experiments from other (11) and our (8,12) laboratories have shown that this increase in CaT is responsible for the second, slow increase in DF observed after stretch. Relatively small increases in Na^+_i can have a large impact on the Ca^{2+} fluxes mediated by NCX. For example, an increase of Na^+_i from 8 to 10 mM would cause a negative shift (-17 mV) of the NCX reverse potential. This change in reverse potential of NCX prolongs the time during which NCX operates in the reverse mode. It might be argued that Na^+/K^+ ATPase activity will compensate any increase in Na^+_i. However, the fact that increasing the beating frequency leads to a rise in Na^+_i that is sustained while high pacing persists (13), would suggest that the increase in Na^+/K^+ ATPase activity is not able to completely buffer an increased Na^+ influx. It is interesting to recall that Bluhm and colleagues (14) showed, using a computerized ionic model of myocardial cells, that a rise in Na^+_i of 2.5 mmol/L increased CaT by ~30%, causing an increase in muscle force comparable to that observed after stretch. These values should, however, be corrected by the subsarcolemmal Ca^{2+} values (which are higher than bulk cytosolic) that are the ones sensed by NCX. Considering the subsarcolemmal Ca^{2+} values, an increase in Ca^{2+} influx through NCX was recently reported in heart failure due to an increse in Na^+_i of ~ 3 mmol/L (15).

The results of Figure 8 illustrate that the above proposed autocrine/paracrine increased formation and release of ET underlies the increase in CaT induced by stretch, since blockade of ET_A receptors eliminates the change in CaT and also the slow increase in DF mentioned before (8,12).

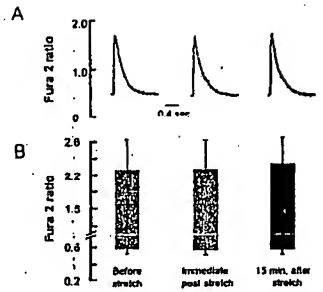

Figure 8. Blockade of ET_A receptors abolishes the changes in CaT induced by stretch. *A.* Representative CaT (assessed by Fura-2 ratio) recorded at 3 different time-points of the stretch protocol. *B.* Averaged (±SEM) values of peak systolic (upper floating columns) and diastolic (bottom floating columns) ratios. Modified with permission from Alvarez et al. (8).

To address whether the increase in CaT results from a decrease of Ca^{2+} extrussion or increased Ca^{2+} entry mediated by NCX, we took advantage of KBR 7943, a selective inhibitor of the reverse mode of NCX (16), at least at concentrations not higher than 5 μM (17). Figure 9 shows that KBR 7943 completely suppresses the slow increase in DF (SFR) after stretch, irrespective of the moment of its application.

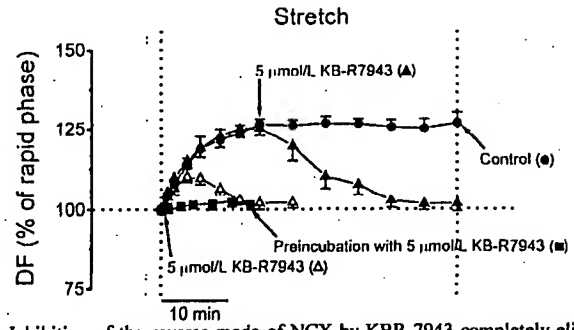

Figure 9. Inhibition of the reverse mode of NCX by KBR 7943 completely eliminates the slow increase in force that follows myocardial stretch. The KBR 7943 effect is independent of whether it is applied prior to or once the contractile response has begun. Modified with permission from Pérez et al. (12).

Given the fact that stretch has distinct effects on pH_i in the presence or absence of bicarbonate and that changes in pH_i modify myofilament Ca^{2+} sensitivity, we sought to explore whether the difference between both experimental conditions can be appreciatted in the SFR to stretch. Figure 10 shows that this is the case because in muscles superfused with HEPES buffer (where pH_i is increased by stretch) the magnitude of the SFR is almost double that elicited under bicarbonate.

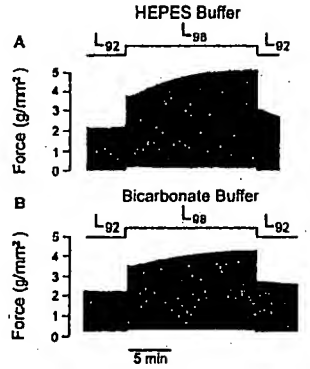

Figure 10. Records of developed force (DF) showing a typical difference in the contractile response to stretch of a papillary muscle superfused with nominally bicarbonate-free (HEPES) buffered medium compared to one bathed with a CO_2/bicarbonate containing medium.

Since CaT increased about the same in both conditions (Figure 11), the greater SFR in HEPES has to be ascribed to an increase in myofilament Ca^{2+} sensitivity due to the rise in pH_i that takes place in the absence of bicarbonate (see Figure 4). A similar increase in CaT is in accordance with the finding that Na^+_i rose by similar amount (Figure 12 and also Figure 4) and would therefore allow to conclude that the lack of pH_i increase under bicarbonate is due to an increase acid load carried by the AE (6,7), and not to a lesser NHE activation.

If our interpretation is correct, eliminating the increase in CaT by KBR 7943 should completely eliminate the SFR in bicarbonate, where the increase in CaT would be the only factor in determining the magnitude of the SFR. To the contrary, KBR 7943 should only partially inhibit the SFR in HEPES, because the sensitizing effect of increased pH_i should persist. The results of such type of experiments are illustrated in Figure 13, which shows that KBR 7943 attenuates the SFR only partially in HEPES but completely in bicarbonate.

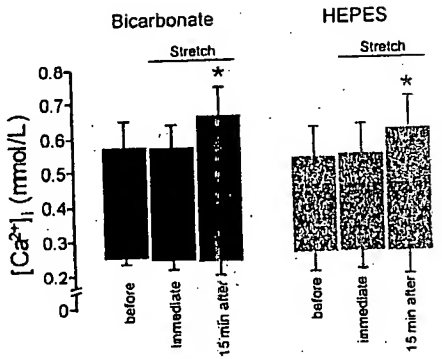

Figure 11. Increases in intracellular Ca^{2+} in bicarbonate- or HEPES-buffered medium. Three different time-points during the stretch protocol are shown. Bottom limits of floating columns: intracellular diastolic (± SEM) Ca^{2+}; upper limits: systolic (± SEM) Ca^{2+}_i values. (*) indicates significant difference compared to values before stretch.

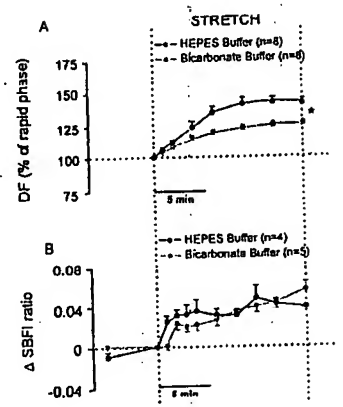

Figure 12. Stretch-induced increases in DF and intracellular Na^+ in bicarbonate- vs. HEPES-buffered medium. (*) indicates significant difference between curves.

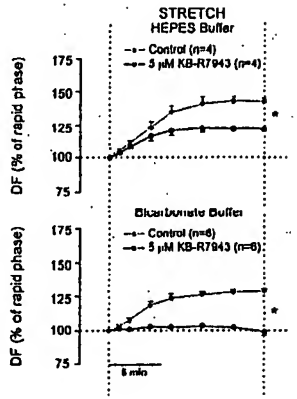

Figure 13. Comparison of the effect of KBR 7943, an inhibitor of the reverse mode of NCX, on the SFR in HEPES- or bicarbonate- HEPES buffered medium. (*) indicates significant difference between curves.

Moreover, a sensitizing effect of increased pH_i under HEPES should prolong twitch relaxation because Ca^{2+} would be more tightly bound to TnC. Figure 14 shows the time to 50 (t_{50}), 67 (τ) and 90 (t_{90}) % of relaxation, as well as the ratio between the maximal velocities of contraction.

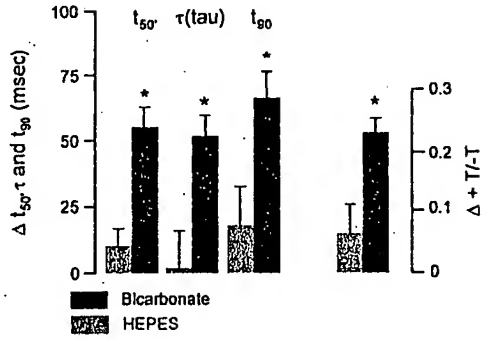

Figure 14. Negative lusitropic effect of stretch under HEPES buffer. All the relaxation parameters examined were increased in HEPES but did not change in the preence of bicarbonate. (*) indicates significant difference compared to bicarbonate.

In summary, the results presented herein support our proposal that in cardiac muscle, mechanical stress elicits an autocrine/paracrine cascade involving Ang II-ET release and stimulation of NHE activity, which subsequently elevates Na^+_i and promotes the reverse mode of NCX. This in turn increases CaT and DF. The magnitude of the increase in force will be determined by the increase of CaT and of pH_i if the latter happens to occur, as in bicarbonate-deprived conditions.

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Horacio E. Cingolani, Néstor G. Pérez and María C. Camilión de Hurtado are Established Investigators of Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

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Chapter 10

THE PARADOXICAL ROLE OF Na^+/H^+ EXCHANGER IN THE DIABETIC HEART

Danielle Feuvray
Physiologie Cellulaire, UMR CNRS 8078, Université Paris-Sud XI, France

1. INTRODUCTION

The idiosyncrasies of nature are often suggestive of much more complex consequences of homeostatic processes than are at first apparent, and Na^+/H^+ exchanger (NHE) activation is no exception. Experimental evidence has shown that stimulation of the exchanger, despite its necessity for intracellular pH (pH_i) restoration after acidosis (see 1-3 for reviews), may contribute to myocardial injury (4). The concomitant influx of sodium ions creates an ionic imbalance that can generate calcium loading *via* reverse sodium-calcium exchange, leading to contractile dysfunction and ultimately cell death (5- 7). NHE activation is therefore a paradoxical phenomenon because it is a major mechanism for restoration of pH_i after an episode of intracellular acidification, but this in turn leads to cell injury.

2. DEPRESSED Na+/H+ EXCHANGER (NHE) ACTIVITY IN INSULIN-DEFICIENT (TYPE 1) DIABETES AND ASSOCIATED INCREASED RESISTANCE OF DIABETIC HEARTS TO ISCHEMIA AND REPERFUSION INJURY

The first evidence that a diseased state such as diabetes may provide an interesting insight into the role of the Na^+/H^+ exchanger in tissue injury associated with cardiac ischemia and reperfusion was published by

Khandoudi *et al.* in 1990 (4). The authors used phosphorus nuclear magnetic resonance spectroscopy to follow the time course of intracellular (pH_i) decline during global ischemia and of recovery on reperfusion in isolated working rat hearts (Figure 1). A unique feature observed was that pH_i recovery occurred significantly more slowly in hearts from chemically (streptozotocin, STZ)-induced diabetic rats than in the normal rat hearts (Figure 1). In comparison, when NHE was pharmacologically inhibited in normal hearts, a slow pH_i recovery was also observed. Moreover, a higher postischemic recovery of contractile function, as assessed by the recoveries of aortic flow and stroke volume, was observed for those hearts with slower pH_i recovery.

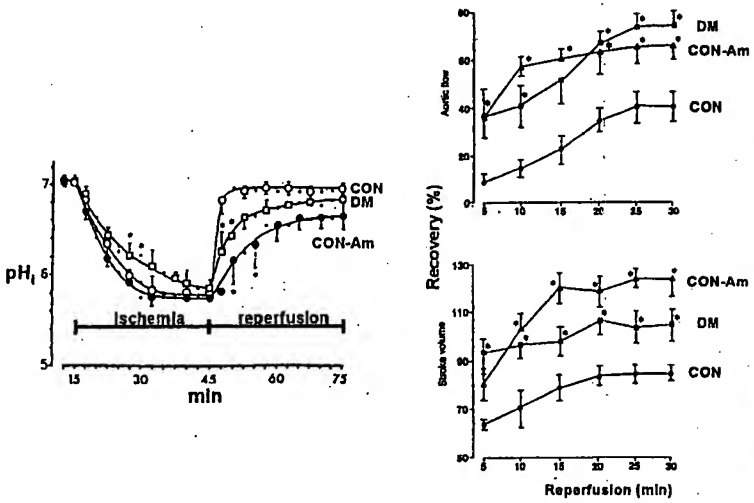

Figure 1. pH_i recovery on reperfusion occurs more slowly in diabetic hearts than in non-diabetic hearts. Left panel: The time course of pH_i decline during ischemia and of recovery on reperfusion was followed by means of ^{31}P-NMR spectroscopy in non-diabetic (CON), diabetic (DM) and non-diabetic hearts with NHE inhibition (CON-Am). Working-heart preparations were submitted to a zero-flow ischemic period of 30 min at 37°C and then reperfused for 30 min. Right panel: Time courses of aortic flow and stroke volume recoveries (expressed as the percentage of the preischemic value) on reperfusion for the three groups of hearts. *P<0.05 vs. non-diabetic hearts. Reproduced in part from Khandoudi et al. (4) with permission.

The observation of a marked decrease in the rate of pH_i restoration following ischemia-induced acidification in diabetic hearts could be put in parallel with earlier results obtained in papillary muscles from STZ-induced

diabetic rats, showing a marked slowing down of the recovery from induced acidosis (8). In addition, differences in pH_i recovery rate between normal and diabetic muscles were abolished by Na^+/H^+ exchange blockade. A striking depression of NHE was shortly after reported in a study in which exchanger activity was measured as intravesicular H^+-dependent Na^+ uptake into isolated sarcolemmal vesicles (9). Since these initial studies that used the STZ-induced model of type 1 diabetes, one article published a few years ago (10) also reported inhibition of Na^+/H^+ exchanger in genetically Bio-Bred (BB/W) diabetic rat hearts. These diabetic rats are indeed considered to be a useful model of autoimmune human insulin-dependent diabetes mellitus (IDDM) (14).

However, the mechanistic basis for the altered activity of the exchanger is still not clearly elucidated. One possibility may be a reduced calcium/calmodulin-dependent kinase II phosphorylation of the protein. Studies have indeed indicated that the control of NHE-1 isoform, representing the major isoform found in the mammalian myocardium (12), may involve intracellular calcium (see 2 for review). Fliegel and coworkers (13) have shown that the purified protein of the cardiac NHE could be directly phosphorylated by calmodulin kinase II. To our knowledge, the first evidence concerning the regulation of the exchanger by this kinase in a cellular system came from investigations in cardiac ventricular myocytes (14). Our data demonstrated that inhibition of calmodulin kinase II under acid load conditions results in a significant reduction in Na^+/H^+ exchange activity. This raises the interesting possibility of basal control (that is to say in the absence of any agonist stimulation) of cardiac NHE activity by direct calmodulin kinase-dependent phosphorylation. Moreover, results suggest that this calcium-dependent regulatory pathway is probably affected by diabetes (14). Alternative or additional possibilities for a decrease in sarcolemmal NHE activity induced by diabetes could be *via* some alterations in intracellular signaling mechanisms. The main trigger for stimulation of NHE activity is cytoplasmic H^+ through an established allosteric mechanism (see 2 for review). It has been hypothesized that through a change in the microenvironment of the exchanger the altered membrane composition (15) may induce a shift in its affinity for intracellular and extracellular H^+. In this context, extracellular H^+ ions have been shown to exert an inhibitory action on the cardiac exchanger (16). Therefore, if diabetes induced a change in the external pH dependence of NHE, this may have at least partly participated in exchanger depression. However, our results did not favour such a hypothesis (14). Interestingly, recent data obtained in cultured neonatal rat ventricular myocytes (17) have suggested that additional regulation to that by H^+ ions of NHE activity may be achieved *via* the extracellular regulated kinase (ERK) pathway. Whether this important regulating pathway can be affected by diabetes has not been investigated so far.

There is now extensive evidence supporting the concept that NHE represents an effective target for pharmacologic intervention for the protection of the ischemic and reperfused myocardium (4,7,10,18,19 and 20 for review). The most convincing experiments used ^{23}Na nuclear magnetic resonance spectroscopy in isolated perfused hearts. These experiments have shown that inhibition of the exchanger markedly attenuated the rise in intracellular sodium during ischemia and reperfusion in non-diabetic hearts (10,18,19) (Figure 2).

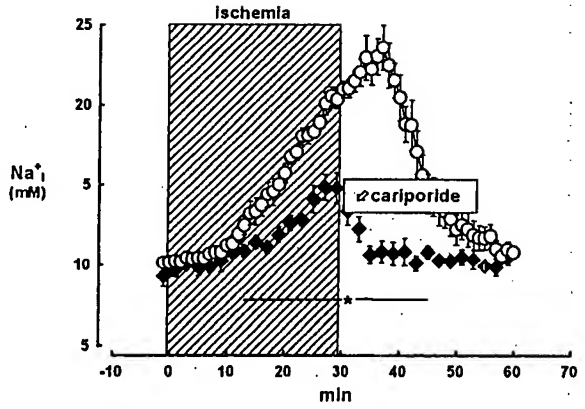

Figure 2. NHE inhibition markedly attenuates the ischemia induced increase in Na^+_i. Mean values of intracellular sodium (Na^+_i) obtained from ^{23}Na-nuclear magnetic resonance spectra, during 30 min of no-flow ischemia and reperfusion for non-diabetic hearts receiving or not receiving cariporide (1 μM). Reproduced in part from Baetz et al. (19) with permission.

However, whereas inhibition of the exchanger attenuated the rise in sodium during ischemia by 154 % in non-diabetic hearts, the reduction was only 64 % in hearts of genetically BB/W rats (10). This observation was in agreement with the observations reported by Imahashi *et al.* (21) using chemically-induced diabetic rats. Furthermore, it is noteworthy that at the end of 30 min of zero-flow ischemia intracellular sodium increase was significantly less (nearly 50 %) in diabetic hearts compared to non-diabetic hearts. These data were consistent with the impairment in Na^+/H^+ exchanger activity. The depression in cardiac NHE activity was determined in ventricular myocytes isolated from chemically (STZ)-induced diabetic rat hearts. To do this acid fluxes through NHE were calculated following

intracellular acidification induced in HEPES-buffered solution, that is to say in the nominal absence of bicarbonate (14). The activity of NHE was found to be markedly decreased over an intracellular pH range similar to that observed during an episode of ischemia in diabetic hearts (22). For example, for a pH_i decrease as low as 0.2 - 0.3 pH units (that is to say $pH_i \approx 6.9$), a reduction of ≈ 42 % in NHE activity was calculated when compared with ventricular myocytes from non-diabetic rats.

It is worth mentioning that data obtained from studies using either isolated ventricular myocytes or isolated perfused hearts showed that diabetes remains without effect on steady-state intracellular pH (4,14,22). Steady-state pH_i in cardiac cells is the result of a balance between the activities of three membrane carriers. Activation of NHE and of Na^+-HCO_3^- cotransport (NBC) induces alkalinization whereas activation of the Cl^-/HCO_3^- exchanger triggers acidification (23). Inhibition of one of these systems would thus lead to a shift in the steady-state pH_i towards either acidic or alkaline values. The Cl^-/HCO_3^- exchange system was shown to participate similarly in recovery from alkalosis in both normal and diabetic rat ventricular myocytes (8), and our earlier work also showed NBC activity to be unchanged (24). Therefore, since NHE activity is impaired by diabetes, HCO_3^- -dependent mechanisms appear to be essential for maintaining steady-state pH_i. Furthermore, our previous data suggested that HCO_3^- -dependent pH_i recovery from an acid load could substitute for the Na^+/H^+ exchanger when the latter is inhibited, as in diabetes. Thus, the contribution of NBC to pH_i recovery from intracellular acidification in diabetic cardiac myocytes was increased up to 38 % at pH_i 6.9 *vs.* 31 % in normal cardiac myocytes. Moreover, the contribution of NBC became predominant even over the NHE contribution at a lower pH_i (*e.g.* pH_i 6.75), reaching ≈ 58 % of the total acid efflux *vs.* 33 % in normal myocytes. This may be particularly important in the initial stage of reperfusion after an ischemic episode.

3. NHE IN NON-INSULIN-DEPENDENT (TYPE 2) DIABETES

As described above, most reports of diabetes-induced cardiac dysfunction, especially concerning the Na^+/H^+ exchanger, have used insulin-deficient (type 1) diabetic animals. To our knowledge, no study has been conducted with non-insulin dependent diabetes mellitus (NIDDM) animal models. However, NIDDM (type 2) comprises the largest group of diabetic patients since it accounts for > 90 % of all cases of diabetes. An increased incidence of cardiovascular diseases is the most common complication in NIDDM (25,26). The cardiac complications associated with NIDDM are due to both increased coronary heart disease secondary to atherosclerosis

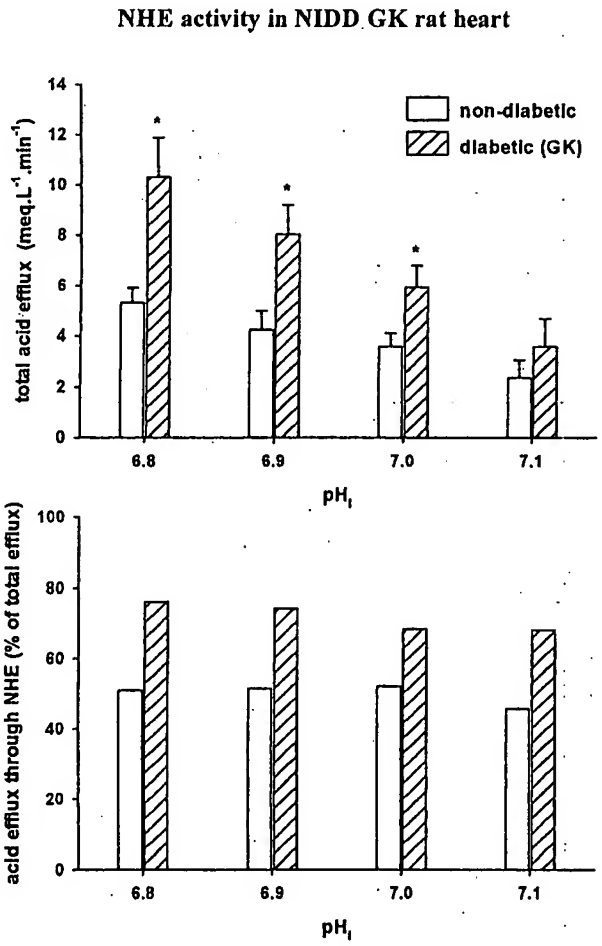

Figure 3 NHE activity in NIDD GK rat hearts. Total acid efflux was determined in experiments using a pH sensitive fluoroprobe with a microepifluorescence technique, in bicarbonate-buffered superfusing solutions. Ventricular myocytes were obtained from hearts of either non-diabetic or diabetic GK rats. Total acid efflux occurred through both NHE and NBC activity. Acid efflux through NHE was calculated by subtracting acid efflux obtained in presence of 1 µM cariporide from total acid efflux. *D. Baetz et al., unpublished data. GK rats were a gift from MERCK-Santé France (D. Mésangeau).*

(27) and a specific diabetic cardiomyopathy resulting in ventricular dysfunction (28).

The Goto-Kakisaki (GK) rat, without any evidence of overt obesity, shows the main features of metabolic, hormonal and vascular disorders usually described in human NIDDM, namely fasting hyperglycemia, pronounced glucose intolerance, peripheral insulin resistance, ...(see 29 for review). We recently examined the activity of NHE in ventricular myocytes isolated from hearts of GK rats (Figure 3). Preliminary (unpublished) data indicate that intracellular pH recovery following intracellular acidification is accelerated in diabetic myocytes in comparison with that observed in myocytes from control rats. Under the experimental conditions used, acid efflux occurred through both NHE and NBC activity. However, by using cariporide to inhibit NHE, it was possible to distinguish between acid efflux carried by each of the two transporters, in other words it was possible to determine NHE activity and NBC activity, respectively, over a given pH_i range.

Results indicate an increase in NHE activity in ventricular myocytes obtained from NIDD GK rats. This is opposite to the decrease in cardiac NHE activity associated with insulin-dependent diabetes in cardiac myocytes (4,8,10,14).

4. CONCLUSION

Much work needs to be done in regard of our recent observations of an increase in NHE activity associated with non-insulin dependent diabetes in GK rats. In particular, future studies should aim at examining whether or not the increase in NHE activity in NIDDM is associated with upregulation of the exchanger and/or increased expression of the protein. Another important aspect to be investigated is whether there could be any possible relationship between NHE activity and the development of a hypertrophic process in NIDDM. Indeed, several recent studies have suggested that the exchanger could play a determinant role in the hypertrophic response (30,31). In this context, it would appear most pertinent to analyze the response of GK diabetic rat hearts to stress situations such as ischemia and reperfusion.

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Chapter 11

ROLE OF Na-H EXCHANGER IN VASCULAR REMODELLING IN DIABETES

Diabetes and vascular disease

Peter J. Little and Rodney J. Dilley

Cell Biology of Diabetes and Morphology Laboratories, Baker Heart Research Institute; Department of Medicine, Monash Universtiy and Alfred Hospital, Prahran, Australia

1. INTRODUCTION

Diabetes is a very significant metabolic disease which is categorised into two major forms – Type 1 and Type 2(1-3). Type 1 is the immunological disease, usually but not always occurring in the young, resulting from an acute failure to produce insulin and requiring life-long insulin treatment for survival (3). Type 2 diabetes is the complex metabolic disturbance associated with dietary factors and sedentary lifestyles leading to obesity and mechanistically resulting from an initial decline of insulin responsiveness ("insulin resistance") and later loss of beta cell function and declining insulin secretion (3, 4). The diagnostic criteria and defining feature of both types is hyperglycemia, although especially in the case of Type 2 diabetes other factors such as hypertension, dyslipidemia and other disturbances such as elevated PAI-1 need to be considered (5). Both forms of diabetes are associated with similar chronic "complications" – mostly micro- and macro vascular disease and related changes (Fig. 1)(6, 7).

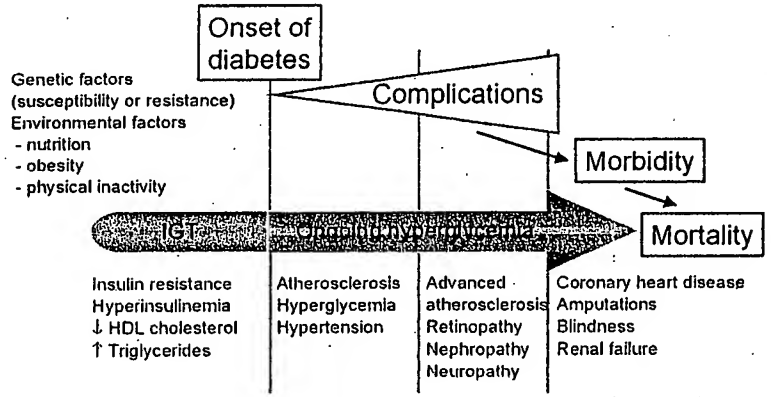

Figure 1. The natural history and progressive nature of the development of the "complications" of Type 2 diabetes. As insulin resistance followed by islet cell failure leads to increasing hyperglycaemia, which cannot be controlled by current therapeutic agents, a progressive development of complications occurs at a rate mostly dependent upon the control of the hyperglycaemia, as well as the control of blood pressure and dyslipidemia and a potentially ameliorative role of unknown protective genes.

Microvascular disease is primarily due to hyperglycaemia and is manifest as specific end-organ damage of nephropathy, retinopathy, neuropathy and impotence (6, 8). Macrovascular disease has a more complex etiology and results from vessel remodelling, excessive lipid penetration and accumulation, acceleration of the formation of atherosclerotic lesions ("plaques"), and with plaque rupture leading to the downstream ischaemic damage of heart attacks, strokes and amputation from coronary, cerebral and peripheral artery disease, respectively (5, 9). The extent of vascular disease depends upon the interaction between the severity and control of the diabetes and the "protection" resulting from critical but unknown genetic factors (10). Coronary heart disease (CHD) manifest as heart attacks is the largest cause of premature death in the general population but diabetes accelerates at least several fold the process of atherosclerosis and CHD becomes the major cause of death with a mortality rate of almost 70—80 per cent (9, 11). Changing work practices and lifestyles associated with economic development have lead to greatly increased rates of obesity and thus diabetes. Overall rates of CHD were falling in the later decades of last

century and this trend has now halted or reversed due to the rising rate of diabetes and its associated high rate of macrovascular disease and CHD (12).

Vascular changes in patients with diabetes occur over long periods of time and can therefore be difficult to study. As such, we have used cell culture and animal models of diabetes to examine early vascular changes that may be the basis of accelerated vascular disease. This chapter focuses on the role of Na/H exchange in these early vascular changes in experimental diabetes. There are six NHE isoforms of which NHE-1, the most widely expressed, is essentially ubiquitous (13). NHE-1 is the only isoform expressed in vascular cells and so this chapter focuses almost exclusively on that isoform (14) and its role in controlling vascular function in a manner that may contribute to or prevent vascular disease in diabetes.

2. MECHANISM AND PROCESSES CONTROLLING THE ACTIVITY OF NHE-1

NHE-1 is a plasma membrane glycoprotein, which is expressed at low abundance (13,15). It has low or negligible basal activity that is regulated in response to the need to expel metabolically derived acid – thus if metabolic activity is low, then Na/H exchange activity is low and vice versa. This is evident when comparing cultured vascular smooth muscle cells from rats and rabbits, where both metabolic activity and Na/H exchange activity are very low in rabbit vascular smooth cells (16) but appreciable in rat cells(17, 18). The major role of NHE-1 as an ion transporter arises from the fact that it is strongly and rapidly activated, providing a great increase in the capacity to extrude an acid load and thus protect against acidosis (18, 19). It is also activated by biochemical pathways that may relate to its role in cell proliferation (14, 20). Essentially the role of Na/H exchange is determined by the multiplicity of factors that regulate its activity, which include:

- Intra and extracellular sodium (21, 22)
- Intracellular protons through the "proton modifier site"(19)
- Extracellular protons (pH)(17, 18)
- Signalling via cell surface receptors such as receptor tyrosine kinases (23, 24), G-protein coupled receptors(25) and integrins (26)
- Glucose as elevated glucose in conjunction with growth factors (27)
- Hyperosmotic stress as a volume regulatory response (28) and

- Negative inhibitory regulation through the binding of calcineurin homologous protein (CHP)(29, 30) and calmodulin (31, 32)

Many pathways of NHE-1 activation involve or pass through the ERK or MAPK pathways (33, 34) however ERK does not directly phosphorylate NHE-1(33). The p90 ribosomal S6 kinase (p90RSK), a serine/threonine kinase, phosphorylates NHE-1 (35) and is most likely the pathway for growth factor activation(20). Importantly, in this context cAMP is not involved in NHE-1 phosphorylation and activation (36). The activation due to hyperosmotic stress occurs independent of phosphorylation (37). Thus, although there has been much work and evidence of phosphorylation the functional outcome and significance of phosphorylation remains unresolved.

Recruitment of molecules to the cell surface appears to be the only major biochemical pathway not to play a role in the regulation of NHE-1 activity since Na/H exchange does not appear to be regulated in this manner, as would insulin-stimulated glucose transporter translocation (38).

Thus, there are a multitude of factors reported to alter, particularly to activate, Na/H exchange but the question is what is the net effect of these specific biochemical alterations on Na/H exchange activity and hence function?

3. CELLULAR PHYSIOLOGY OF THE ACTIVATION OF NHE

Na/H exchange can be activated by a variety of pathways and this has been the subject of considerable research as outlined above – initial suggestions that a simple model of phosphorylation may be operative (39) have proven to be more complex with multiple factors and now co-factors controlling activity(40). The factors that activate NHE have been extensively reviewed (41) however, the cellular physiology of the meaning of "activation" has often not been clearly enunciated or defined. This may be particularly relevant to a glycoprotein such as NHE-1 that is subject to hormone and growth factor mediated phosphorylation, other biochemical changes and stimuli such as acidosis or osmotic stress. Furthermore, the multi-functional nature of the NHE-1 possessing external and internal sodium and proton binding sites, an internal proton sensor associated with its ion translocation function suggests further layers of complexity. Traditional models of the activation have suggested a classic horizontal "shift to the right" of the activation curve relating intracellular pH and NHE activity such that the actual ion translocation activity at any particular intracellular pH (pH_i) is increased but the minimum and maximum are unaltered. It should be noted that the direction, to the right or left, might depend upon whether the

proton concentration is expressed as molar or pH but the functional consequence of higher activity at the same pH_i remains. However, we suggest that the data (25) and indeed our own findings(42) indicate that it is more appropriate to define the cell physiology of NHE activation as an "upward" shift in the activity to pH_i relationship – that is, not only is the ion translocation activity at any pH_i increased but both the maximum activity is increased, and the minimum or basal activity ("set point") is increased giving a higher resting pH_i (Fig. 2).

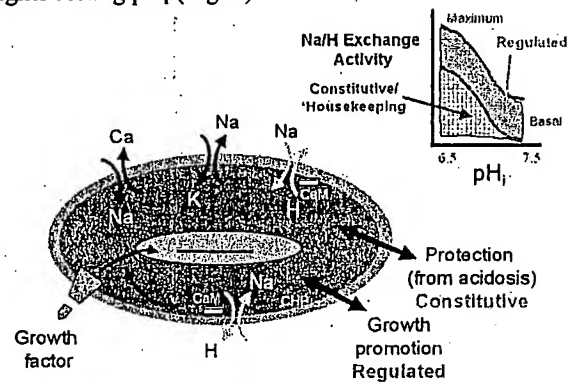

Figure 2. Proposed model of "constitutive" and "regulated" Na/H exchange activity in vascular smooth muscle cells. The constitutive activity relates to the basal metabolism and need for pHi homeostasis. A multiplicity of pathways activate Na/H exchange leading to an effective "upward shift" (see text) in the activity to pHi relationship with higher levels of activity at lower levels of pHi. The consequence is that activated Na/H exchange activity is expressed as both an elevated maximal activity and elevated basal pHi

Work from Barber's laboratory examining the activity of NHE driven by constitutively activated G proteins clearly shows that constructs with greater NHE activity have higher basal pH levels (25). This is important in examining changes in NHE activity since it implies that true activation arising from the action of growth factors or similar stimuli will cause an increase in maximal activity and an increase in basal pH_i.

The consequence of our proposal is that a full and proper evaluation of the impact of external stimuli on the activity of Na/H exchange requires determination of the maximum activity, the minimum activity or effectively the basal pH_i at which the exchanger is not operative and the slope of the relationship between activity and pH_i as a measure of the sensitivity to protons.

4. ION TRANSPORT AND CYTOSKELETON IN THE CELLULAR FUNCTIONS OF NHE1

The distribution of NHE1 and other plasma membrane ion transport proteins is not uniform across the cell surface but is localised to form microdomains, which have specific local transport functions (43, 44). The domains relate to membrane anchoring points of the cytoskeleton and thus a novel area of investigation is emerging to define the ion transport/cytoskeleton interactions and their role in regulation of volume, shape, adhesion and motility of cells (45). It has been recognized that ion channel cytoskeleton binding regulates both the localization and activity of ion transport, and conversely, that ion transport activity may mediate cytoskeleton-dependent cell functions (44).

Ion channel proteins contain binding regions that interact with the cytoskeleton through linker proteins (e.g. ankyrin, the ezrin-radixin-moesin (ERM) proteins, 4.1 ERM and spectrin), which form complexes similar to the integrin binding complex on cytoskeletal actin filaments (46). Mutations to the linker binding regions of NHE1, independent from mutations to the transport region, have been shown to reduce cell polarity and prevent forward motion and disassembly of the trailing edge in migrating fibroblasts (41). Binding of ion channel proteins to complexes containing tubulin may also suggest formation of microtubule-based cytoplasmic microdomains (47) or important regulatory transport mechanisms(48).

The relationship between integrin binding to extracellular matrix ligands and activation of NHE1 is well known (49). Integrins and NHE do not have a specific structural association, however in NHE1 null mutant fibroblasts the assembly of integrin-mediated focal adhesions is reduced, but rescued by stable expression of wildtype NHE1 or of a mutant with intact cytoskeletal linker protein interactions and no transport activity (50, 51). It has been suggested that NHE1 is able to establish an anchorage-dependent assembly of the cytoskeleton that allows cell adhesion.

The spatial relationship between cytoskeleton membrane anchoring proteins and ion channel proteins suggests a functional relationship. NHE1 activity is localised in a polarised fashion in migrating cells as application of NHE1 inhibitor to lamellipodia is able to inhibit migration, but application to the cell body does not (52). The contribution of the NHE1 activity to solute uptake and cell swelling at the leading edge of cells is not known. NHE1 ion transport activity is not required for cytoskeletal tethering, as mutants lacking ion transport activity retain tethering capacity and bind to ERM proteins (50), but de-adhesion and retraction of the trailing edge are impaired(53). Similar tail-end polarization of K channel activity is thought to regulate volume changes in migrating cells (54). NHE1 mutations specifically blocking ERM binding and cytoskeletal anchoring display a

different phenotype, with impaired polarity at the leading edge and persistence of a primary lamellipod. Actin cytoskeleton is disorganised in the primary lamellipod suggesting that NHE1 tethering capacity has an important cytoskeletal organisation role and may define cytoskeletal polarity in some microdomains. It will be interesting to determine if normal physiological regulation of NHE expression or localization will be able to regulate cellular functions relying on changes in cell shape or motility, such as migration.

4.1 Vascular Changes In Diabetes

Vascular smooth muscle has been the subject of much work in the area of Na/H exchange, perhaps because of the importance of vascular smooth muscle cell proliferation in the aetiology of vascular disease, a major disease burden in modern society and the focus of intense research activity (14, 31, 55). NHE has been investigated in a variety of vascular smooth muscle preparations from cells to vessels (17, 42, 56, 57). Vascular tissues have also proven to be highly suitable for radiosodium influx studies and fluorescent-dye based studies of intracellular pH (17, 18). Our early work showed close correlation of results from cultured vascular smooth muscle cells and isolated intact vessels, thus validating cultured cell models in relation to vascular changes *in vivo* (17). Seminal studies showed the key role of Na/H exchange in vascular smooth muscle cell growth in that fully competent mitogens caused activation of NHE activity, whereas agents causing cellular hypertrophy but not hyperplasia did not activate NHE activity (14, 39, 58). Furthermore, original studies showing the inhibitory effects of amiloride analogues on fibroblasts, previously reported to occur only under bicarbonate-free conditions (59), when repeated in vascular smooth muscle cells showed that the amiloride analogues were effective inhibitors of cell proliferation under bicarbonate buffered conditions(58). This latter study provided initial indications of mechanisms by demonstrating a cell cycle specific effect of ethylisopropylamiloride to inhibit the latter of two phases of de novo protein synthesis that occurred during the G1 phase of the cell cycle in cells exposed to serum born mitogens (58).

Of relevance to diabetes, it was shown that NHE expression and activity was acutely and chronically regulated by glucose concentration in the culture media when altered to mimic the hyperglycaemia of diabetes (27). These experiments were undertaken in the presence of serum-derived growth factors with the implication that the high glucose may have its major effect by stimulating or synergizing with the effects of growth factors rather than there being a glucose-only stimulatory pathway. We recently showed that the

stimulation of vascular smooth muscle cell growth by PDGF is specifically activated under high glucose conditions (60).

Scheid and colleagues investigated the role of Na/H exchange in blood vessels in hypertension (57, 61) finding that Na/H exchange activity was elevated in the mesenteric arterioles of spontaneously hypertensive rats compared to Wistar Kyoto controls and further that the induction of hypertension does not per se increase Na/H exchange activity in vessels (61). We set out to follow up studies in cultured cells by us (17, 18, 58) and others(27) to investigate the role of Na/H exchange in the development of vascular hypertrophy in diabetes. Models of diabetes which present or respond similarly to the human condition have been difficult to develop and they may need to differ conceptually to model either Type 1 or Type 2 diabetes. With the defining factor of elevated blood glucose - hyperglycaemia – the model of the streptozotocin-induced hyperglycaemic, often referred to as a diabetic rat, has been well characterised and widely used. Adult rats respond to a single injection of the toxic streptozotocin with an increase in blood glucose levels over several days to those levels experienced transiently by a person with poorly controlled diabetes (20-25mM glucose). Vascular changes in the mesenteric bed have been characterised by the work of Cooper and colleagues who have shown the lumenal expansion and increase in cross-sectional area of the third order mesenteric vessels which occurs several weeks after the induction of hyperglycaemia (62, 65). The diabetic rats become hyperphagic, which is a possible contributor to the hypertrophy in mesenteric vessels, but hyperphagia and vascular hypertrophy can by dissociated by the use of angiotensin converting enzyme (ACE) inhibitors. Vascular hypertrophy is prevented by ACE inhibitor treatment whilst the hyperphagia persists (63). The mechanism of the vascular hypertrophy in this model involved cell proliferation and matrix deposition (62, 63).

Streptozotocin-treated animals have profound hyperglycaemia throughout their vascular compartment and based on the studies of Williams *et al.* (27) it would be predicted that the entire vascular tree would undergo activation of Na/H exchange activity and as a consequence vascular smooth muscle cell proliferation and vascular hypertrophy. However, some evidence suggests that not all vascular beds hypertrophy in response to streptozotocin-induced diabetes. Moore *et al.* reported that rat cerebral vessels do not hypertrophy in response to hyperglycaemia (66). We undertook to investigate the hypertrophy and Na/H exchange in basilar arteries. Hypertrophy can be assessed by weighing the mesenteric tree but the cerebral vessels are too small to apply this simple technique – following streptozotocin-induced hyperglycaemia there was no increase in mesenteric weight at one week but a 56 % increase at 3 weeks (42). Using quantitative morphometry on both vascular beds we found an increase in the cross sectional area of the lumen, media and adventitia of the mesenteric vessels at

3 weeks but no changes were apparent in the cerebral vessels confirming the earlier reports (Little, unpublished observations)(63, 66). Mesenteric hypertrophy in this model was manifest as an increase in vessel diameter and in total cross sectional area. Conveniently, basilar arteries are of a similar diameter (100-300μm) to the mesenteric vessels allowing for the investigation by concomitant myography and fluorescence for the monitoring of intracellular pH in the media of the vessels. The fluorescence obtained from dye-loading an intact microvessel is derived from the media and the endothelium does not play a significant role in the fluorescence recorded (C. Aalkjaer, personal communication). Mesenteric and cerebral arterioles were mounted in a myograph and loaded with the cell permeable, fluorescent pH-sensitive dye, BCECF-AM, and placed through a contraction protocol to equally work all vessels and remove work related acidosis as a confounding parameter. All experiments were conducted in HEPES buffered, bicarbonate-free solutions to remove the impact of bicarbonate transporters. Mesenteric arterioles had elevated basal pH_i and maximal Na/H exchange activity by one week, that is, before the hypertrophy had commenced and the elevated pH_i and maximal Na/H exchange activity was maintained through at least 3 weeks (42). Under identical experimental conditions there were no changes in any of the parameters used to assess the activation status of the Na/H exchanger in cerebral vessels (Little, unpublished observations). Interestingly however the maximum rate of Na/H exchange in the control animals was 50 per cent higher in the cerebral compared to the mesenteric vessels. We speculate at this stage that the level of activity reflects the basal level of expression of NHE1 and Na/H exchange protein necessary to maintain pH homeostasis, but that it is the changes in pH_i and maximum activity that represent the activity of intravascular growth factors mediating the biochemical mechanisms of growth outlined earlier. To assess the role of Na/H exchange in mediating the hypertrophic effects and the potential to prevent the vascular changes, a further group of animals was subject to treatment with the Na/H exchange antagonist, cariporide or its vehicle. Cariporide had very small effects on the weight of the mesenteric beds from the control animals but appreciably and significantly reduced the hypertrophy. We did not attempt to assess the level of Na/H exchange activity in the presence of the inhibitor. The most interesting result arose from histological staining of the vessel. Although the basic study was premised on the ability of a Na/H exchange inhibitor to prevent vascular smooth muscle cell proliferation, it was found that the hypertrophy was due to an increase in matrix deposition and this deposition was greatly reduced by cariporide treatment (42).

It was surprising that the major effect of cariporide was on matrix deposition. Although the cariporide study most likely related to collagen accumulation, other matrix molecules may also be affected. We have been

investigating the potential role of proteoglycans in atherogenesis (67, 68). We and others have shown that growth factors regulate the biosynthesis of proteoglycans, and importantly the length of the glycosaminoglycan side-chains which bind Low Density Lipoproteins (69-71); cardiovascular and anti-diabetes (72) drugs can reverse the growth factor mediated glycosaminoglycan elongation and reduce LDL binding (73, 74). In view of the effect of cariporide on matrix in vivo we recently examined the effect of ethylisopropylamiloride and HOE 694 on proteoglycan biosynthesis by primate vascular smooth muscle cells under normal glucose conditions and under high glucose conditions which mimic the hyperglycaemia of diabetes. Intriguingly both agents inhibited the synthesis of vascular proteoglycans and we are presently pursuing this interesting observation (Fig. 3).

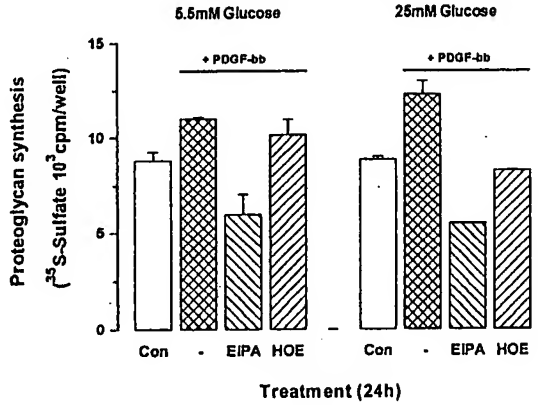

Figure 3. Inhibition of vascular smooth muscle cell proteoglycan biosynthesis by Na/H exchange inhibitors. Monkey aortic smooth muscle cells were grown to confluence, serum deprived then metabolically labelled with 35S-sulfate for 24 hours in DMEM with 0.1% serum and in the presence of ethylisopropylamiloride EIPA (40 μM) or HOE 694 (100 μM) and platelet derived growth factor (20 ng/ml) as indicated under low (5.5 mM) and high (25 mM) glucose conditions. The media was harvested and proteoglycans isolated by CPC precipitation (69, 74)

4.2 Future Prospects

The latest work suggests that Na/H exchange may have two possibly independent roles being the plasma membrane ion translocation function and a cytoskeletal anchoring function. Clearly a variety of inhibitors are available to inhibit the ion translocation function and it might be contemplated that agents which interfere specifically with the cytoskeletal role of Na/H exchange might be developed since it is likely that the

functional domains will be distinct. Whether the role of cytoskeletal antagonists would extend beyond an experimental tool is problematical however migration of cells, which might be affected by such an agent, is important in several settings including the development of a neo-intima in response to vascular injury (75).

Our findings of the role of cariporide in inhibiting diabetic vascular hypertrophy by a mechanism involving reduced matrix deposition requires further consideration. Matrix is a vital component of vascular structure and function and that the inhibition was observed with a newer generation inhibitor (which are more specific in their actions than the amiloride derivatives) suggests that there may be a relationship between excess matrix secretion and Na/H exchange. Such an activity would have the potential to generate a new therapeutic target for Na/H exchange inhibitors beyond the role so far established in cardiac protection (76). So, new generation highly specific low toxicity Na/H exchange inhibitors may well be worth investigation as a new pathway to the prevention of vascular disease in diabetes.

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Chapter 12

THE POTENTIAL ROLE OF THE Na^+/H^+ EXCHANGER IN ISCHEMIA/REPERFUSION INJURY OF THE CENTRAL NERVOUS SYSTEM

John W. Phillis[1], Julie G. Pilitsis[2] and Michael H. O'Regan[3]
[1]Department of Physiology, [2]Department of Neurological Surgery, School of Medicine, Wayne State University, Detroit, Michigan, USA. [3]Biomedical Sciences, School of Dentistry, University of Detroit Mercy, Detroit, Michigan, USA

1. INTRODUCTION

The Na^+/H^+ exchangers (NHEs) are a group of expressed membrane proteins which have a role in regulating intracellular pH. NHEs are expressed in virtually all mammalian cells and there are currently eight identified isoforms (NHE1-NHE8). The exchangers, together with other membrane transport systems such as the Na^+/HCO_3^- cotransporter and the Cl^-/HCO_3^- exchanger, provide an important mechanism for eliminating excessive acid production during physiological cell metabolism and under pathological conditions (1). NHEs 6-8 differ from the other isoforms in that they are found intracellularly, where they may associate with mitochondria and the trans Golgi network rather than with the plasma membrane (2).

NHEs are present in both neuronal and glial cells (3). NHE-1 is the most prominently expressed isoform in the central nervous system (CNS) (4) where it appears to play an important role in the regulation of intracellular pH and cell volume under both normal and pathological conditions (5). Expression of the NHE-1 isoform becomes apparent in the CNS during the first postnatal week (5). NHE fluxes are based on the transmembrane gradients of Na^+ and H^+ and are not directly dependent on ATP to function,

although the latter is required to maintain a physiological transmembrane Na^+ gradient. During ischemia-evoked intracellular acidosis plasma membrane NHEs are activated and extrude H^+ ions in exchange for the uptake of Na^+ in an attempt to stabilize intracellular pH. The effect of NHE activation is electroneutral (6) and appears to be regulated by a number of mechanisms (7). These include: phosphorylation of the NHE cytoplasmic domain by protein kinases C and A, increases in cytoplasmic calcium, and activation by Ca^{2+} and GTP-binding proteins.

Indications of the physiological role of NHEs in nervous function have been gained from studies on knockout NHE-1 mice. These animals display locomotor ataxias and tend to develop seizures. CA1 hippocampal neurons in NHE knockout mice had a more acidic intracellular pH under physiological conditions and a slower recovery of intracellular pH following an acidic load (5).

2. Na^+/H^+ EXCHANGE, STROKE AND POTENTIAL ADVERSE CONSEQUENCES

Acidosis, which occurs during ischemia, can contribute to cell swelling in part as a result of the activation of NHEs (8,9). In this instance, the increase in intracellular Na^+, together with its osmotically obligated water, initiates cell swelling which can be inhibited by exposure to amiloride, an NHE blocker (8). Swelling, in turn, is associated with the release of osmolytes, including the excitotoxic amino acids glutamate and aspartate, from neurons and glia through swelling-activated plasma membrane anion channels as part of a regulatory volume decrease (RVD) phenomenon (10,11).

Another potentially unfortunate consequence of NHE activation with elevated levels of intracellular Na^+ during ischemia is the entry of Ca^{2+} via a reversed action of the Na^+/Ca^{2+} exchanger (NCX) which extrudes Na^+ ions from the cell in exchange for Ca^{2+} (12). This calcium overload can lead to a variety of deleterious effects including: mitochondrial accumulation of calcium, activation of proteolytic enzymes and lipases including phospholipases A and C, with lipolysis and free radical formation (7,12). The loss of calcium homeostasis and increase in intracellular calcium in ischemia is a critical factor in the chain of events leading to cellular death (13).

2.1 Acidosis And Ischemic Brain Injury

The contributions of acidosis to ischemic brain injury have been controversial. Although severe acidosis has been linked to increased cell damage, there is also evidence that milder acidosis can have cerebroprotective effects. The acidotic injury hypothesis has been challenged by the observation that at the pH typically generated in ischemic brain, currents coupled to the NMDA receptor were almost entirely abolished (14,15). As NMDA receptors appear to be critically involved in excitotoxic ischemic injury (16) these findings suggested that acidosis could actually limit excitotoxic injury. Mild acidosis was subsequently shown to protect hippocampal and cortical neurons from ischemia-simulating conditions (14,17), and cortical and cerebellar cultures from glutamate toxicity (18,19). Furthermore, brain acidosis induced by hypercarbic ventilation attenuated focal ischemic injury *in vivo* (20) and hypoxic-ischemic damage to the immature rat brain (21). The potential for acidosis to be cerebroprotective was subsequently referred to as a "paradoxical wrinkle" (22).

Bond et al. (23) used the term "pH paradox" to describe the protective actions of intracellular acidosis on cultured cardiac myocytes exposed to simulated ischemia, proposing that the injury actually occurs during the recovery to normal pH. They suggested that although favorable conditions for the activation of degradative enzymes such as proteases and phospholipases exist during ischemia, the acidotic environment inhibits their stimulation. As the pH rapidly recovers during reperfusion, damage to mitochondrial and plasma membranes occurs, initiating necrotic or apoptotic processes (24) with the ultimate fate – "necrosis or apoptosis" – of the cell depending on the extent of mitochondrial loss and ability to restore ATP levels.

Support for the conjectures of the Lemasters group can be found in studies on the effects of hyperglycemia on stroke injury. Hyperglycemia enhances injury when it is induced prior to but not after ischemia (25-28). Hyperglycemia increases infarct size in collaterally perfused but not end-arterial vascular territories (29), and in permanent focal ischemia, hyperglycemia may be protective (30,31). These findings suggest that the harmful effects of glucose in stroke may depend critically upon the degree to which collateral perfusion is available to the specific brain regions affected, the extent to which blood flow is reduced, and the timing of glucose administration. The mechanism(s) underlying hyperglycemia-enhanced cerebral ischemic injury remain uncertain. A rise in lactate production and concomitant tissue acidosis has been proposed as an explanation (32,33). Measurement of intracellular pH values during 15 min of ischemia followed

by reperfusion in moderately hypoglycemic and hyperglycemic rats demonstrated that during hypoglycemia pH_i declined to ~6.35 whereas in hyperglycemia it was <6.00 (32). Recovery was rapid in both sets of animals during the initial 5 min of reperfusion, with further normalization in the subsequent 25 minutes. pH_i in the hyperglycemic animals then tended to rebound beyond the initial pH_i value of ~7.15. As phospholipase A_2 activity is very sensitive to pH, with maximal activation at a slightly alkaline pH (34) it is apparent that the onset of acidosis would limit the activity of this enzyme, with recovery during the reperfusion-induced rebound of pH.

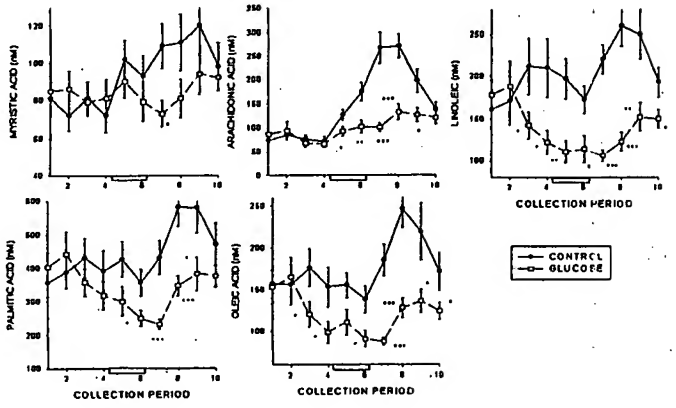

Figure 1. Effects of hyperglycemia on ischemia-evoked release of FFAs into cerebral cortical superfusates. Line plots show the time course of changes in superfusate FFAs before, during, and following a 20-min period of four vessel occlusion (collections 5 and 6, open box). D-Glucose (3.4 g/kg) was administered prior to the start of collection 3. Data are presented as means ± SEM. Statistically significant differences between superfusate FFAs in normo- and hyperglycemic animals were determined by a two-tailed Students t-test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Reproduced with permission from Phillis et al. (36).

Evidence in support of an involvement of PLA_2 in ischemic injury has been obtained from *in vivo* studies on the formation and release of free fatty acids in the ischemic rat cerebral cortex. A 20 min period of four vessel occlusion-induced ischemia evoked an initial small increase in the efflux of arachidonic, myristic, palmitic, linoleic and oleic acids into cortical superfusates (which was significant only for arachidonic acid) from the rat cerebral cortex followed by a decline in release (35). Reperfusion was

associated with rapid, pronounced, increases in FFA release, which were significant for arachidonic, linoleic and oleic acids, followed by a slow decrease during the subsequent 40 min. When similar experiments were conducted on hyperglycemic rats (3.4 g/kg 30 min prior to ischemia; plasma glucose at onset of ischemia 603 ± 144 mg/dl and 218 ± 43 mg/dl at onset of reperfusion) there were declines in the basal levels of some FFAs and significant reductions in the effluxes of arachidonic, myristic, palmitic, linoleic and oleic acids during ischemia/reperfusion in comparison with saline injected controls (36; Figure 1).

These findings are consistent with the Bond et al. (23) proposal of a "pH paradox" effect with hyperglycemia-evoked acidosis having a potent inhibitory effect on phospholipase A_2 which was sustained into the reperfusion period. Subsequent studies with selected inhibitors of the various forms of PLA_2 have confirmed that a calcium-dependent PLA_2, which is selectively inhibited by $AACOCF_3$, is involved in the FFA release (37).

2.2 Inhibition Of NHEs During Cerebral Ischemia/ Reperfusion

A majority of the research on the effects of intracellular acidosis on ischemia/reperfusion injury is to be found in the cardiovascular literature. Bond et al. (23) proposed that it is the return to normal pH during reperfusion which is responsible for the loss of cell viability and subsequent death. Thus the worsening of injury, termed the "pH paradox", is mediated by the recovery of intracellular pH.

Recovery of intracellular pH may be detrimental to cells for a number of reasons. Ischemia can activate Ca^{2+}-dependent hydrolytic enzymes such as phospholipases and proteases, whose activity will initially be inhibited by the reduced pH environment, and then restored to full activity during reperfusion. The change from acidosis to normal pH during reperfusion may also induce a pH-dependent mitochondrial permeability pore transition (MPT), resulting in a futile cycling of Ca^{2+} across the mitochondrial inner membrane which compromises the ATP supply (24).

Acid efflux from cells across the plasma membrane via the Na^+/H^+ exchanger plays a large role in the recovery of normal pH (38,39). Inhibition of the Na^+/H^+ exchanger has proven to be an effective method for delaying pH recovery during the ischemia/reperfusion sequence. The selective Na^+/H^+ exchange inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA) protected cultured cerebellar granule cells against glutamate-induced delayed neuronal death (18) and a related compound, dimethylamiloride,

(DMA) protected dissociated cortical cultures during recovery from metabolic inhibition (40). Amiloride derivatives are known to have an affinity for NMDA receptors (18,41), raising the possibility that these agents could have exerted their effects by reducing glutamate toxicity. Vornov et al. (40) attributed the protective effects of DMA to inhibition of Na^+/H^+ exchange with slowing of pH recovery, and argued that rapid pH recovery was correlated with injury, as demonstrated by lactate dehydrogenase release. Ferimer et al. (42) have demonstrated the ability of another amiloride derivative (methylisobutyl amiloride), which did not change intracellular pH from its normal, non-ischemic, value, to significantly reduce cardiac pH_i in rats subject to arrest with 15 min of reperfusion.

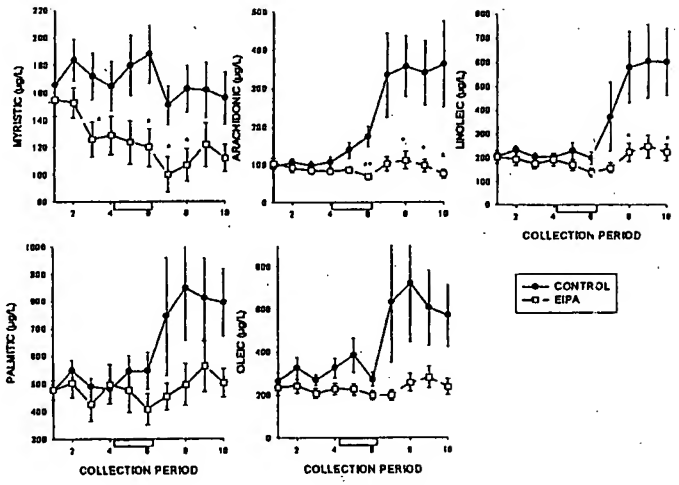

Figure 2. The effect of EIPA (25µM; added to aCSF prior to collection 3) on ischemia-evoked FFA release into rat cerebral cortical superfusates. Line plots show the time course of changes in superfusate FFAs before, during, and following a 20-min period of four vessel occlusion (collections 5 and 6, open box). Statistically significant differences between control ischemic and drug treated animals were determined by a two-tailed Students t-test. * $p<0.05$; ** $p<0.01$. Reproduced with permission from Phillis et al. (45).

The severity of cerebral ischemic damage has been shown to directly influence the rate of free fatty acid accumulation (43,44). The pronounced increase in FFA levels in rat cerebral cortical superfusates during reperfusion (45) indicated that phospholipase activity was being affected by the sequential changes in intracellular pH. This concept was tested by slowing

the recovery of pH with two NHE inhibitors, EIPA and the more potent and selective non-amiloride SM20220. When applied topically, both compounds significantly reduced FFA formation and release (Figures 2 and 3) (45,46).

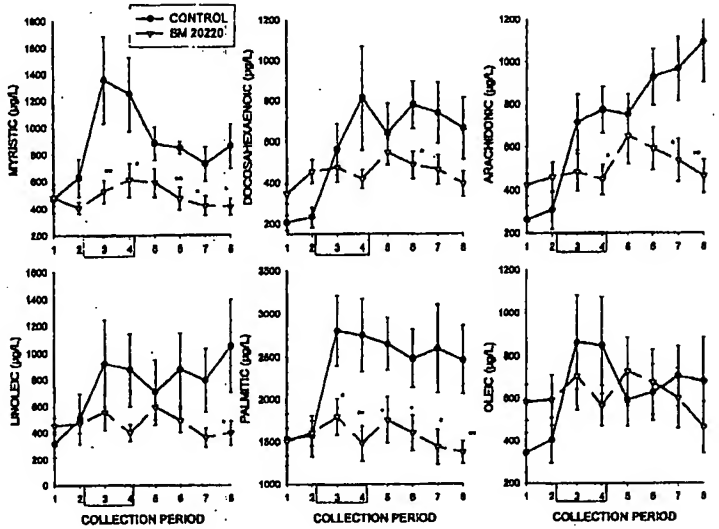

Figure 3. The effect of SM-20220 (20µM, added to aCSF prior to the start of collection 2 and present throughout all subsequent collections) on ischemia-evoked FFA release into rat cerebral cortical superfusates. Line plots show the time course of changes in superfusate FFAs before, during, and following a 20-min period of four vessel occlusion (collections 3 and 4, open box). Statistically significant differences between control ischemic and SM-20220 animals were determined by a two-tailed Students t-test. * $p<0.05$; ** $p<0.01$. Reproduced with permission from Pilitsis et al., 2001a.

EIPA also protected gerbil CA1 hippocampal pyramidal neurons from ischemic injury (47). Similar effects of SM20220 have been reported in the gerbil hippocampal ischemia model (48) and in a rat middle cerebral artery occlusion model (49).

Harmaline inhibits an amiloride-insensitive NHE isoform (50) in hippocampal neurons which is likely an NHE-5 variant (51). Although this compound had protective effects on the recovery of neuronal cultures from metabolic inhibition (40), when applied topically at a 500 µM concentration it did not attenuate free fatty acid efflux from the rat cerebral cortex during ischemia/reperfusion (Pilitsis J.G. et al., unpublished observations). This observation suggests that it is the NHE-1, rather than the NHE-5, isoform

that is involved in limiting phospholipase activity either through maintaining acidosis or limiting increases in intracellular calcium.

As well as delaying pH recovery, evidence suggests that as these inhibitors limit the increases in intracellular Na^+ they also reduce increases in intracellular Ca^{2+} resulting from a reversed action of the Na^+/Ca^{2+} exchanger which extrudes Na^+ ions in exchange for Ca^{2+} (12,52,53). The calcium overload can lead to a variety of deleterious effects including activation of phospholipases, with lipolysis, and free radical generation, as well as activation of proteases. Elevations in intracellular Ca^{2+} can precipitate a further accumulation of this ion by mitochondria with activation of the MPT and bioenergetic failure (54).

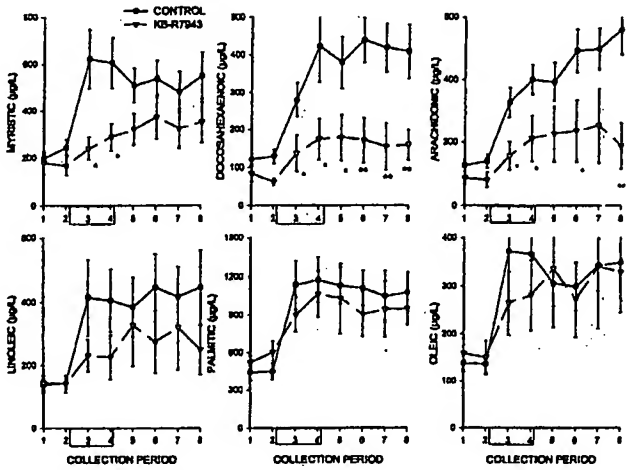

Figure 4. The effect of KB-R7943 (50μM) on ischemia-evoked FFA release into rat cerebral cortical superfusates. See legend to Fig. 3 for further details. Reproduced with permission from Pilitsis et al. (56).

Inhibition of the Na^+/Ca^{2+} exchanger (NCX) with KB-R7943, a specific inhibitor of the reversed mode of the NCX, has been shown to be neuroprotective in a hippocampal slice model of ischemia/reperfusion injury (55). When applied topically onto the rat cerebral cortex, KB-R7943 attenuated the ischemia/reperfusion-evoked release of phosphoethanolamine and FFAs (Figure 4) indicating that it had decreased Ca^{2+} entry into the cytoplasm via the Na^+/Ca^{2+} exchanger, with a reduction in Ca^{2+}-dependent

phospholipase activity (56). Another Na^+/Ca^{2+} exchange inhibitor, SEA 0400, attenuated reperfusion injury in a rat middle cerebral artery occlusion model (57).

Inhibition of mitochondrial Na^+/Ca^{2+} exchange by CGP-37157 (50 μM), a specific inhibitor of this enzyme, strongly attenuated free fatty acid efflux from the ischemic/reperfused rat cerebral cortex (58). Previous studies had shown that increases in PLA_2 activity occur following Ca^{2+} release from mitochondria (59,60). Inhibition of Ca^{2+} release by CGP-37157 would therefore be expected to attenuate phospholipase activity and the release of FFAs. Studies with PLA_2 knockout mice have shown a decrease in infarct size and improvement of neurological outcome following ischemia/reperfusion (61).

3. CONCLUSION

The data presented in this report demonstrate that NHE plays an important role in the development of ischemia/reperfusion injury in the central nervous system. The problem appears to stem from the exchanger's ability to effect a rapid recovery of intracellular pH to physiological levels, activating already Ca^{2+} primed phospholipases and proteases which can result in damaging consequences to neurons and glia. An important role of the phospholipases A_2 in the breakdown of plasma and mitochondrial membranes, with the formation of free fatty acids is apparent from the data. FFA oxidation by cyclooxygenases and lipoxygenases can subsequently lead to the formation of free radical and potentially damaging eicosanoids. The success of NHE inhibitors in protecting the brain from ischemia/reperfusion injury in animal studies suggests that these compounds may be of therapeutic value for the treatment of stroke and cardiac arrest patients. Future studies with more selective agents will allow a better determination of whether the protective effects of NHE inhibition are a consequence of maintaining acidosis and limiting increases in intracellular calcium with reductions in the activation of Ca^{2+}-dependent phospholipases and proteases, and if other mechanisms of action are also involved.

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Chapter 13

RECEPTOR-MEDIATED REGULATION OF THE CARDIAC SARCOLEMMAL Na^+/H^+ EXCHANGER

Mechanisms And (Patho)Physiological Significance

Robert S. Haworth and Metin Avkiran
Centre for Cardiovascular Biology and Medicine, King's College London, The Rayne Institute, St Thomas' Hospital, London, UK

1. INTRODUCTION

Intracellular pH (pH_i) homeostasis in cardiac myocytes is achieved principally by the integrated action of 4 different sarcolemmal ion transporters (1). When the myocyte cytoplasm becomes acidic, the Na^+/H^+ exchanger (NHE) and the Na^+/HCO_3^- cotransporter (NBC) extrude acid from the cell, while under conditions of intracellular alkalosis, the Cl^-/HCO_3^- and Cl^-/OH^- exchangers effectively import acid. In order to investigate the function and regulation of NHE, experimental protocols are often performed in the absence of bicarbonate, which renders NBC inactive and thereby makes NHE the sole acid extrusion pathway. NHE activity is regulated primarily by pH_i, and increases markedly in response to intracellular acidosis (1) through the interaction of H^+ with an allosteric modifier site on the transport domain (2,3). The basal activity of the sarcolemmal NHE is low under physiological conditions, while increasing intracellular acidosis leads to a pH_i-dependent increase in NHE activity, with a Hill coefficient of around 3 (4). This indicates that more than 1 proton binds to the NHE protein during the transport cycle, and has led to the suggestion that the NHE protein contains a non-transporting proton-binding site which allosterically modifies NHE activity. Thus, as pH_i falls, the proton modifier site becomes increasingly occupied, leading to a greater increase in NHE activity than

would be expected by simply increasing the availability of transportable protons.

The cardiac sarcolemmal NHE is encoded by the NHE1 gene (5), and appears to be localised predominantly to the intercalated disc region and transverse-tubules of the myocyte (6). The protein consists of a 500 amino acid transport domain and a 300 amino acid regulatory domain. The proton modifier site is found in the transport domain and is functional in the absence of the regulatory domain; nevertheless, the maintenance of pH_i in the normal range depends upon the interaction of the regulatory domain with the transport domain (7). The regulatory domain of NHE1 contains a number of potential phosphorylation sites (7). Activation of several kinase pathways leads to an increase in NHE1 phosphorylation, and a corresponding increase in NHE activity (8,9). It has been suggested that phosphorylation of the regulatory domain may cause a conformational change in the protein, altering the way in which this domain interacts with the transport domain and possibly increasing the affinity of the allosteric modifier site for protons, thus increasing transport activity (2). The list of putative NHE1 kinases now includes Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (10), extracellular signal-regulated kinase (ERK) (11), the 90 kDa ribosomal S6 kinase ($p90^{RSK}$) (12), p38 mitogen-activated protein kinase (p38 MAPK) (13), and Nck-interacting kinase (NIK) (14). It should be noted, however, that removal of the last 100 or so amino acids from the regulatory domain eliminates NHE1 phosphorylation, but results in only a 50 % inhibition of NHE activation in response to growth factor and thrombin stimulation, indicating that other factors are involved in regulating NHE activity (2,15). Deletion experiments have suggested that the binding of Ca^{2+}/calmodulin to the regulatory domain increases NHE1 activity (3,16,17). In contrast, the binding of calcineurin-homologous protein (CHP) to a separate site may inhibit NHE1 activation (18). However, more recent data have suggested that CHP may be an essential co-factor for NHE1 activity (19). Notably, much of the work on the molecular mechanisms that regulate NHE1 activity has, by necessity, been carried out in non-cardiac cells (which are more readily amenable to molecular manipulation, for example by transfection), and the relative importance of these mechanisms in regulating native NHE activity in the cardiac sarcolemma remains unclear.

2. REGULATION OF CARDIAC SARCOLEMMAL NHE ACTIVITY BY RECEPTOR ACTIVATION

Considerable evidence has now accumulated that the sarcolemmal NHE of cardiac myocytes is subject to regulation by a variety of stimuli that act through G protein-coupled receptors (GPCRs).

2.1 Adrenergic Receptors

The majority of studies on the regulation of sarcolemmal NHE activity by neurohormonal stimuli have been focused on catecholamine-induced signaling, with particular attention on the role of α_1-adrenoceptors (α_1-ARs). In isolated ventricular myocytes from the rat, norepinephrine caused a rapid increase in resting pH_i which was inhibited by the α_1-AR antagonist prazosin and by 5-N,N-hexamethylene amiloride, an inhibitor of NHE (20). Also in rat ventricular myocytes, exposure to the selective α_1-AR agonist phenylephrine induced intracellular alkalinisation (21). In this study, alkalinisation was inhibited both by prazosin and by ethylisopropylamiloride, another inhibitor of NHE. Wallert and Fröhlich reported similar results using the α_1-AR agonist 6-fluoronorepinephrine (22). In this latter study, an agonist-induced increase in both resting pH_i and in the rate of recovery of pH_i following an acid load was observed. In guinea-pig ventricular myocytes, phenylephrine increased the rate of recovery of pH_i following an acid load (23). Again, the effect of phenylephrine was inhibited by both prazosin and the NHE inhibitor amiloride. These studies provide strong evidence that α_1-AR stimulation leads to increased sarcolemmal NHE activity.

Until recently, it has not been possible to identify which subtype(s) of the α_1-AR is/are responsible for the stimulation of NHE activity. Three subtypes of the α_1-AR have been identified pharmacologically (α_{1A}, α_{1B} and α_{1D}), and mRNA transcripts for all three are detected in the heart (24). Using α_1-AR antagonists that have different selectivity for the α_1-AR subtypes, we have shown that α_1-adrenergic stimulation of NHE in adult rat ventricular myocytes is likely to be mediated via the α_{1A}-AR subtype (25). This was confirmed by demonstrating that A61603, an α_{1A}-AR-selective agonist, could also increase sarcolemmal NHE activity.

We have recently shown that the mechanism by which α_{1A}-AR activation leads to an increase in NHE activity involves both protein kinase C (PKC) and the ERK pathway (26). Inhibition of PKC with GF109203X or the ERK pathway with PD98059 blocked α_1-AR-mediated stimulation of NHE activity. However, inhibition of PKC did not inhibit ERK activation by

phenylephrine or A61603, indicating that α_1-AR-mediated ERK activation works through a PKC-independent pathway, but that activation of both PKC *and* ERK is required for the α_1-AR-mediated increase in NHE activity.

In considering adrenergic regulation of the sarcolemmal NHE, it is important to note that β_1-adrenergic receptors (β_1-ARs), which are normally more abundant than α_1-ARs in myocardium, also regulate NHE activity. Indeed, there is a degree of agreement among published studies that, in contrast to α_1-adrenergic stimulation, β_1-adrenergic stimulation inhibits NHE activity in some species. In guinea-pig ventricular myocytes, isoprenaline slowed pH_i-recovery after an acid load in HCO_3^--free solution (23). Isoprenaline also caused a fall in resting pH_i in sheep Purkinje fibres, again in HCO_3^--free solution (27). In dog Purkinje fibres, no effect of isoprenaline on resting pH_i was observed, but recovery from an acid load was slowed, again indicating that β-adrenergic stimulation inhibited NHE activity (28). In this study, pretreatment with atenolol was shown to abolish the inhibition of NHE activity by isoprenaline, confirming that this response is mediated by the β_1-AR.

In the dog Purkinje fibre, the inhibitory effect of β_1-AR stimulation could be mimicked by treatment with the adenylate cyclase activator forskolin, or the phosphodiesterase inhibitors theophylline and 3-isobutyl-1-methylxanthine (IBMX), indicating a role for cAMP (28). Similarly, IBMX inhibited NHE activity in the sheep Purkinje fibre model (29). Mechanistically, it is not clear how elevation of cAMP leads to the inhibition of NHE activity. Although a regulatory role for protein kinase A (PKA) is well established for NHE3, there are no consensus phosphorylation sites for PKA in the regulatory domain of NHE1, and it is unlikely that direct phosphorylation of NHE1 by PKA occurs (30). Although activation of an inhibitory NHE1 kinase by PKA cannot be ruled out as a contributory mechanism, it is intriguing that trifluorperazine, a non-specific Ca^{2+}-calmodulin inhibitor, is able to block the inhibitory effect of IBMX (29).

The net effect of the endogenous catecholamines, norepinephrine and epinephrine, on sarcolemmal NHE activity may depend on the relative density or availability of α_1- and β_1-ARs, which in turn may explain age- and species-specific differences in the response of NHE activity to catecholamines, as well as altered responses in disease.

2.2 Angiotensin II Receptors

Angiotensin II is a peptide hormone which produces positive inotropic and chronotropic effects in several mammalian species. A possible role for NHE in mediating the effect of angiotensin II on the L-type Ca^{2+} channel

current was suggested in rabbit ventricular myocytes (31), although an effect on NHE activity was not demonstrated directly. In the perfused ferret heart, the application of angiotensin II caused an increase in pH_i through activation of NHE (32). This activation was inhibited by GR-117289, a selective non-peptide inhibitor of the angiotensin II type 1 (AT_1) receptor. A very small (but significant) effect of angiotensin II on resting pH_i was demonstrated also in adult rat ventricular myocytes (33), with the authors concluding that angiotensin II caused an increase in sarcolemmal NHE activity. More recently, we have shown that angiotensin II, when used alone, has no effect on the recovery from an acid load in rat ventricular myocytes (34). However, when given together with PD123319 (an AT_2 antagonist), angiotensin II was able to stimulate sarcolemmal NHE activity in a concentration-dependent manner. Angiotensin II had no effect on NHE activity in the presence of losartan (an AT_1 antagonist), while CGP42112A (an AT_2 agonist) was also without effect. These data suggested that angiotensin II-induced activation of AT_1 receptors stimulates sarcolemmal NHE activity, but that simultaneous activation of AT_2 receptors counteracts this stimulation. The stimulation of NHE activity by angiotensin II plus PD123319 was inhibited by PD98059 (an inhibitor of the ERK pathway), GF109203X (a PKC inhibitor) and tyrphostin AG1478 (an epidermal growth factor receptor [EGFR] kinase inhibitor). The exposure of myocytes to angiotensin II plus PD123319 caused an increase in ERK activity, which was also inhibited by PD98059, GF109203X and tyrphostin AG1478. Thus, AT_1-mediated activation of sarcolemmal NHE requires ERK activation, possibly through a PKC and EGFR-mediated mechanism. The mechanism by which AT_2 stimulation counteracts NHE activation by AT_1 receptors is not known, but does not appear to involve direct inhibition of NHE or attenuation of ERK activation.

2.3 Endothelin Receptors

Endothelin is a 21 amino acid vasoactive peptide, which has a potent positive inotropic effect in mammalian hearts. In adult rat ventricular myocytes, endothelin increased both resting pH_i (33,35) and the rate at which pH_i recovers following an acid load (33), while in canine cardiac Purkinje fibres, endothelin caused a large increase in the rate of recovery following an acid load and a modest alkalinisation of pH_i (28). In rat ventricular myocytes, the endothelin-mediated increase in resting pH_i was inhibited by amiloride (suggesting a role for the sarcolemmal NHE) and by H-7 and sphingosine (indicating a signaling role for PKC) (35). In canine Purkinje fibres, however, pretreatment with H-7 or staurosporine had no effect on endothelin-induced stimulation of NHE activity, suggesting that

PKC is not involved in this preparation (28). Pretreatment of rat ventricular myocytes with pertussis toxin had little effect on the endothelin-induced stimulation of sarcolemmal NHE activity (35); thus, it is likely that, in these cells, the pertinent endothelin receptor couples to PKC through $G\alpha_q$ rather than $G\alpha_o$ or $G\alpha_i$. There is no direct evidence on which endothelin receptor subtype is involved in the regulation of NHE activity. Nevertheless, inhibition of NHE prevented the deleterious effects of endothelin during ischemia and reperfusion in rat hearts, and these effects were also inhibited by PD 155080, a selective antagonist of the ET_A receptor subtype (36). Thus, the ET_A receptor is likely be responsible for endothelin-induced stimulation of sarcolemmal NHE activity, which is consistent with data that this receptor is the predominant endothelin receptor subtype expressed in rat ventricular myocytes (37).

2.4 Thrombin Receptors

Thrombin induces a variety of cellular responses through receptor-mediated pathways, in addition to its well established role in blood coagulation and thrombus formation (38). With respect to the cardiac myocyte, we have shown that both thrombin and the synthetic thrombin receptor activating peptide SFLLRN increase sarcolemmal NHE activity in adult rat ventricular myocytes (39). The common ability of thrombin and SFLLRN to stimulate sarcolemmal NHE activity is consistent with this action being mediated by the thrombin receptor that was first cloned by Vu and collegues (40), which is now known as protease-activated receptor 1 (PAR1) and whose mRNA we have shown to be expressed by adult rat ventricular myocytes (39). Targeted disruption of the PAR1 gene in mice revealed that additional thrombin receptors exist (41). Subsequently a second thrombin receptor, PAR3, has been cloned and shown to be present in human myocardium (42). In addition, PAR2, a trypsin-activated receptor (which is activated by SFLLRN) has been shown to be present in neonatal rat ventricular myocytes (43). Therefore, at present, there is some doubt as to which receptor(s) mediate the thrombin-induced stimulation of sarcolemmal NHE activity. With respect to the pertinent signaling pathways, thrombin-induced stimulation of sarcolemmal NHE activity was inhibited by the PKC inhibitors GF109203X and chelerythrine, which again suggests a mediatory role for this kinase in the regulation of sarcolemmal NHE activity (39).

2.5 Adenosine Receptors

We have recently shown that activation of the adenosine A_1 receptor leads to reduced stimulation of sarcolemmal NHE activity by the α_1-AR agonist phenylephrine (44). This inhibitory effect could be prevented by preincubation of myocytes with either the selective A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine or pertussis toxin, the latter indicating a G_i protein-mediated mechanism. The distal mechanism(s) by which adenosine A_1-receptor occupation inhibits the stimulation of NHE activity by α_1-AR activation is not clear. A_1 receptor activation alone was not sufficient to significantly inhibit basal NHE activity, suggesting that attenuation of the α_1-adrenergic response is unlikely to reflect functional antagonism through an independent pathway. Rather, it appears that A_1-receptor stimulation may initiate events that interfere with the NHE-regulatory signaling mechanisms that lie downstream of the α_1-AR. Intriguingly, the NHE-stimulatory effect of thrombin was similarly inhibited by adenosine A_1 receptor stimulation (44), suggesting a broader inhibitory cross-talk between the G_i protein-coupled adenosine A_1 receptor and multiple G_qPCRs.

2.6 Muscarinic Receptors

In view of the established existence and multiple known functions of muscarinic receptors in myocardium (45), there is surprisingly little information available on the role of these receptors in the regulation of sarcolemmal NHE activity. In canine Purkinje fibres, the application of carbachol, a non-hydrolysable muscarinic receptor agonist, has been shown to increase both resting pH_i and the rate at which pH_i recovered following an acid load (28). These effects were inhibited by both amiloride and atropine, suggesting that carbachol stimulates sarcolemmal NHE activity via muscarinic receptors. The effect of carbachol on NHE activity was inhibited by staurosporine, suggesting an involvement of PKC (28); however, in view of the broad spectrum of action of this kinase inhibitor, the relevant signaling mechanism(s) remain to be confirmed.

2.7 "Orphan" Receptors

Of the 300 or so GPCRs identified through genomic research, endogenous ligands for around 140 receptors are not yet known. It is likely that several of these "orphan" receptors are expressed in the cardiac myocyte, and that some of these may regulate sarcolemmal NHE activity

and thereby cardiac function. Indeed, apelin, the recently identified endogenous ligand of the formerly orphan APJ receptor, has now been shown to have a positive inotropic effect in adult rat heart. This positive inotropic effect of apelin could be partially blocked by pretreatment of hearts with methylisobutylamiloride or zoniporide, inhibitors of the sarcolemmal NHE (46). These data suggest that APJ receptors may stimulate sarcolemmal NHE activity; nevertheless, such a role for APJ receptors remains to be confirmed by direct measurements of NHE activity, and the pertinent signaling mechanisms also await identification.

3. PHYSIOLOGICAL IMPORTANCE OF RECEPTOR-MEDIATED REGULATION OF THE SARCOLEMMAL NHE

The main function of the sarcolemmal NHE is to remove acid equivalents from the cell. Under physiological conditions (i.e. in the presence of bicarbonate), however, NBC activity also contributes to such acid extrusion (1). Therefore, the overall effect of receptor stimulation on the rate of acid extrusion will depend on the responses of both NHE and NBC to the relevant stimulus. Notably, however, there is only limited information available on receptor-mediated regulation of NBC activity. Lagadic-Gossmann *et al* (23) have shown that, in guinea-pig ventricular myocytes, adrenergic stimulation has opposing effects on NHE versus NBC activity; thus, α_1-AR stimulation was shown to activate NHE but inhibit NBC, while β_1-AR stimulation inhibited NHE but activated NBC. In the same study, the net effect of the physiological adrenergic agonists norepinephrine and epinephrine, which stimulate both α_1- and β_1-ARs, was to *slow* the rate of recovery of pH_i following intracellular acidosis in bicarbonate-containing medium. In contrast to the counter-regulatory effects of adrenergic stimulation on NHE versus NBC activity, recent evidence suggests that AT_1 receptor stimulation by angiotensin II increases sarcolemmal NBC activity (47), as it does sarcolemmal NHE activity (34). In the case of NBC, however, the AT_1 receptor-mediated response does not appear to be subject to inhibition via the AT_2 receptor (47). For the variety of other ligands that have been shown to regulate NHE activity (see above), however, information on their effects on NBC activity and the net rate of acid extrusion in bicarbonate-containing medium is not available. Furthermore, little is known about receptor-mediated regulation of the activity of the two sarcolemmal acid loaders, the Cl^-/HCO_3^- exchanger and the Cl^-/OH^- exchanger (48,49). Clearly, the sustained effect of any given stimulus on pH_i will be determined by its net effect on acid equivalent flux across the sarcolemma, as determined by the sum of its actions on inward or outward flux through all four pH-regulatory transporters. Nevertheless, as proposed by Leem *et al* (1), a receptor-mediated shift in the pH_i sensitivity (and therefore the activity) of an individual transporter, such as the sarcolemmal NHE, is likely to produce at least a transient displacement of pH_i, within a permissive range (pH_i 6.95-7.25 in the guinea pig ventricular myocyte).

An increased sarcolemmal NHE activity that arises from receptor stimulation would of course produce not only an increased rate of acid extrusion (and thereby relative intracellular alkalinization) but also an increased rate of Na^+ influx into the myocyte. Each of these processes can have a significant effect on myocardial contractility, through distinct mechanisms. Altered pH_i can regulate contractility at almost every stage of the excitation-contraction coupling cascade, with intracellular alkalosis producing a positive inotropic effect that is mediated largely through increased myofilament Ca^{2+} responsiveness (see review by Orchard and Kentish (50)). In parallel with this, any increase in the intracellular Na^+ concentration ($[Na^+]_i$) that arises from increased Na^+ influx via the sarcolemmal NHE can produce a positive inotropic effect, principally through the modulation sarcolemmal Na^+/Ca^{2+} exchanger activity and thereby $[Ca^{2+}]_i$ regulation (51). Indeed, a variety of neurohormonal stimuli, such as α_1-adrenergic stimulation (21,52), angiotensin II (53,54) and endothelin (35,55) have been shown to produce positive inotropic responses in myocardium that are sensitive to inhibition by inhibitors of the sarcolemmal NHE. Interestingly, the positive inotropic effect of α_1-adrenergic stimulation has been shown also to be inhibited by stimulation of the adenosine A_1 receptor (56), in a manner that parallels the inhibitory effect of this receptor on α_1-AR-mediated stimulation of sarcolemmal NHE activity (44). These data suggest that receptor-mediated regulation of sarcolemmal NHE activity makes an important contribution to the inotropic responses that are produced by a variety of neurohormonal stimuli; nevertheless, the relative contributions of altered pH_i (and thereby myofilament Ca^{2+} responsiveness) versus altered $[Na^+]_i$ (and thereby $[Ca^{2+}]_i$ regulation) have not been clearly demonstrated in each case. Interestingly, the slow increase in force development that is observed in response to myocardial stretch has also been shown to be sensitive to inhibition by NHE inhibitors (57). There is recent evidence that this slow force response occurs through autocrine/paracrine mechanisms that are mediated by the release of angiotensin II and endothelin, which then produce increases in sarcolemmal NHE activity and $[Na^+]_i$ via the stimulation of their cognate AT_1 and ET_A receptors (58).

4. PATHOPHYSIOLOGICAL IMPORTANCE OF RECEPTOR-MEDIATED REGULATION OF THE SARCOLEMMAL NHE

Receptor-mediated regulation of the sarcolemmal NHE is likely to be of importance also in pathophysiological processes where exchanger activity plays a causal or permissive role.

4.1 Receptor-Mediated Regulation Of NHE In Myocardial Ischemia And Reperfusion

Since the late 1980s, substantial evidence has accumulated that increased activity of the sarcolemmal NHE contributes to the development of myocardial injury and dysfunction during ischemia and reperfusion (59,60). Indeed, selective NHE inhibitors have been shown to be cardioprotective in this setting in numerous animal studies (see reviews by Avkiran (61) and Karmazyn *et al.* (62)). Importantly, recent data suggest that NHE inhibition may provide cardioprotective benefit in high-risk patients who undergo global myocardial ischemia and reperfusion during coronary artery bypass graft surgery (63), an observation which awaits confirmation by current clinical trials (64).

It is well established that myocardial ischemia results in activation of the sympathetic nervous system, as well as the local release of norepinephrine within the ischemic zone (65). This appears to be accompanied by enhanced α_1-adrenergic signaling, through increased receptor density and enhanced receptor coupling to distal steps in the relevant signaling cascade (66). It is likely therefore that α_1-AR stimulation may play an important role in regulating sarcolemmal NHE activity during ischemia. In this context, it is important to note that α_1-AR stimulation appears to retain its ability to increase sarcolemmal NHE activity even in the presence of extracellular acidosis, which accompanies myocardial ischemia (67). Furthermore, work in Karmazyn's laboratory has shown that, in isolated rat hearts subjected to global ischemia, α_1-AR stimulation exacerbates post-ischemic contractile dysfunction, through mechanisms that are counteracted by NHE inhibition (68). In our own laboratory, we have shown that, in rat hearts, the incidence of arrhythmias observed during reperfusion following regional ischemia is reduced by the selective NHE inhibitor HOE694 (69). Additionally, we have shown that α_1-AR stimulation selectively in the ischemic zone exacerbates reperfusion-induced arrhythmias, with this pro-arrhythmic effect abolished not only by α_{1A}-AR antagonism but also by NHE inhibition (70). These data indicate that α_1-AR-mediated stimulation of sarcolemmal NHE activity may significantly influence the outcome of myocardial ischemia and reperfusion.

Other receptor-mediated stimuli that alter sarcolemmal NHE activity are also likely to be of pathophysiological importance during myocardial ischemia and reperfusion. Intracoronory thrombosis, which is the commonest cause of acute myocardial ischemia in man (71), is associated with elevated levels of thrombin in the vicinity of the evolving thrombus (72). Thus, thrombin may increase sarcolemmal NHE activity through the stimulation of its cognate receptors. Indeed, thrombin receptor stimulation has been shown to exacerbate the increase in $[Na^+]_i$ that occurs during ischemia (73), although the role of NHE activity in mediating this response has not been determined. Nevertheless, there is preliminary evidence that thrombin induces a potent pro-arrhythmic effect during regional ischemia and reperfusion in the rat heart, by a mechanism that is blocked by NHE inhibition (74). Endothelin is also believed to be released during myocardial ischemia and reperfusion (75), and may lead to the stimulation of sarcolemmal NHE activity. Certainly, exposure of hearts to exogenous endothelin during ischemia has been shown to depress the post-ischemic recovery of contractile function, an effect which could be blocked by treatment with an NHE inhibitor (36,76).

In view of the apparent detrimental effects of receptor-mediated stimuli that increase sarcolemmal NHE activity, it is tempting to speculate that other receptor-mediated stimuli that suppress NHE activity (in the absence or presence of excitatory stimuli) may have the opposite effect, affording myocardial protection during ischemia and reperfusion. Consistent with this, there is evidence that cardiac-specific overexpression of the adenosine A_1 receptor in transgenic mice preserves myocardial integrity and function following regional ischemia and reperfusion (77); nevertheless, whether this effect is mediated through a reduced sarcolemmal NHE activity is not known.

4.2 Receptor-Mediated Regulation Of NHE In Myocardial Hypertrophy

There is substantial evidence that G_qPCR signaling pathways contribute significantly to the development of cardiac hypertrophy, as well as its decompensation that can lead to heart failure (78). In *in vitro* studies, many G_qPCR agonists that stimulate sarcolemmal NHE activity (e.g. α_1-adrenergic agonists, endothelin, angiotensin II, thrombin; see earlier) have been shown also to induce myocyte hypertrophy (79-82). This raises the question of whether the association between the stimulation of NHE activity and the induction of hypertrophy is of a causal or casual nature. Recent data obtained with specific NHE inhibitors may suggest the former. Thus, the hypertrophic

response of adult rat ventricular myocytes to adrenergic stimulation has been shown to be attenuated by NHE inhibition (83,84). Intriguingly, hypertrophic responses to the sustained (24 h) stimulation of either α_1-ARs or β_1-ARs were both attenuated by NHE inhibition (84), despite the fact that acute β_1-AR stimulation *reduces* sarcolemmal NHE activity (see earlier). Furthermore, the ventricular hypertrophy that develops spontaneously in transgenic mice with cardiac-specific overexpression of the β_1-AR has been shown recently to require NHE activity, on the basis that the hypertrophic phenotype was almost completely abolished by chronic treatment with the NHE inhibitor cariporide (85). These observations raise the possibility that NHE activity may play a permissive role in the development of cardiac hypertrophy, such that in the absence of such activity (i.e. in the presence of NHE inhibitors) the phenotype is attenuated regardless of the nature of the hypertrophic stimulus. This is consistent with increasing evidence that NHE inhibitors suppress the development of hypertrophy in a diverse range of *in vitro* and *in vivo* models. Thus, in recent studies, NHE inhibitors have been shown to attenuate stretch-induced hypertrophy of cultured neonatal rat ventricular myocytes *in vitro* (86), post-myocardial infarction myocardial hypertrophy in rats *in vivo* (87-90), and myocardial hypertrophy in response to left (91) or right (92) ventricular pressure overload in rats *in vivo*. It appears therefore that, at least in the rodent heart, NHE activity plays a fundamental role in the induction and evolution of myocardial hypertrophy (see Chapter 14). Nevertheless, the relative importance of increased NHE activity (e.g. in response to G_qPCR stimulation) and the precise molecular mechanism(s) through which NHE activity regulates the induction and/or evolution of myocardial hypertrophy remain to be elucidated.

5. CONCLUSION

Sarcolemmal NHE activity in cardiac myocytes is subject to regulation by a variety of GPCRs. This regulation usually manifests as an increase in NHE activity (e.g. in response to stimulation of α_1-ARs, and endothelin, angiotensin AT_1 and thrombin receptors), although some GPCR systems have been shown to reduce sarcolemmal NHE activity (e.g. β_1-ARs) or to attenuate its stimulation (e.g. adenosine A_1 and angiotensin AT_2 receptors). Receptor-mediated regulation of sarcolemmal NHE activity is likely to play significant roles in both physiological and pathophysiological processes, in particular in the regulation of myocyte contractility and the modulation of myocardial responses to ischemia and reperfusion and a variety of hypertrophic stimuli. Greater understanding of the pertinent signaling mechanisms may lead to the development and/or application of therapies targeted at these mechanisms.

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Chapter 14

ROLE OF NHE-1 IN CARDIAC HYPERTROPHY AND HEART FAILURE

Morris Karmazyn
Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada

1. INTRODUCTION

Heart failure has become an immense medical problem which is reaching epidemic proportions. Death rates from heart failure more than doubled in the past 10 years. In the United States alone, there are almost 5,000,000 individuals who have heart failure with more than a half-million new cases diagnosed each year. Currently, the 5-year mortality rate for heart failure is about 50 percent. In addition to mortality from pump failure, these patients exhibit an incidence of sudden cardiac death at 6–9 times the rate of the general population. In developing strategies for the treatment of heart failure, it is important to appreciate that this is not a disease per se but rather a complex clinical syndrome which is the final common pathway for numerous cellular and molecular defects caused by many instigating factors, the most common of which being myocardial infarction. Indeed, improved survival rates in patients who have had a myocardial infarct represents one of the major reasons for the tremendous increase in heart failure although other factors such as an aging population also represent important contributing factors. As a result of advances in molecular and cellular biology, it is now known that heart failure extends beyond abnormal heart function and organ physiology but involves numerous intracellular defects. Moreover, the complexity of the heart failure process is well-known, particularly in view of the numerous cellular and molecular changes that are seen in heart failure many of which appear to be interrelated. Two important components underlying heart failure include the initial adaptive hypertrophic

response which follows myocardial injury and the second, the subsequent evolution to heart failure (1). Indeed, inhibition of the early (mal)adaptive hypertrophy is an important therapeutic component which can result in an attenuation of the heart failure response. NHE-1 represents a key intracellular pH regulatory process in the cardiac cell after induction of acidosis (2). Emerging evidence suggests that it may play an important role in cell growth (2). In this review the basis for NHE-1 involvement in the heart failure process is discussed as is the experimental evidence from both in vitro and in vivo studies that NHE-1 inhibition prevents both the maladaptive remodelling resulting in heart failure as well as heart failure itself.

2. RATIONALE FOR NHE-1 INVOLVEMENT IN CARDIAC HYPERTROPHY AND HEART FAILURE

There are a number of lines of evidence suggesting that NHE-1 may represent a key factor mediating hypertrophic responses, especially after myocardial infarction, and thus suggesting that the exchanger could be an important cellular target for attenuation of both the hypertrophic responses as well as heart failure. As already discussed in this volume in a number of chapters, particularly Chapters 9 and 13, from a theoretical perspective, it is important to indicate that NHE-1 stimulation can occur through receptor-dependent mechanisms. As illustrated in Figure 1, this reflects the fact that the antiporter is the target of multiple signalling pathways such as those activated by various kinases and G protein-coupled receptors (3, 4). The intracellular pathways leading to activation of NHE-1 are not well understood. Increasing evidence suggests that NHE-1 activation through signalling mechanisms is dependent on mitogen-activated protein (MAP) kinases especially in response to growth factors which are potential candidates as hypertrophic agents. Indeed, NHE-1 possesses consensus sequences for MAP kinase and various studies have implicated MAP kinase in NHE-1 phosphorylation and activation (5). Recently a role for p90rsk in endothelin-1-induced MAP kinase-dependent phosphorylation of NHE-1 has been demonstrated in rat myocardium (6). In addition, protein kinase C activation may represent an important mechanism in the hypertrophic and remodelling process particularly in response to various paracrine and autocrine factors such as endothelin-1, angiotensin II and α_1 adrenergic agonists. Thus, there appears to be a casual relationship between hypertrophic factors and their ability to activate NHE-1 in the sense that a large number of these agents exhibit this property. In addition, as discussed in Chapter 9, stretch-induced activation of receptors leads to stimulation of NHE-1 activity which then contributes to hypertrophic responses.

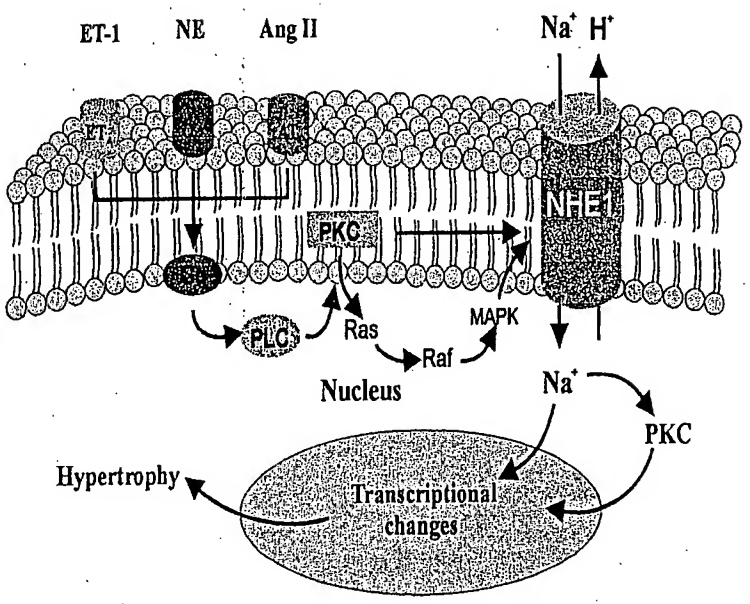

Figure 1. Simplified schematic showing the potential role of NHE-1 in mediating the hypertrophic effects of autocrine/paracrine receptor activation in the cardiac cell. Activation of receptors leads to Gq and PLC dependent PKC and MAPK activation which in turn stimulates NHE-1 activity. The basis for the resultant transcriptional changes leading to hypertrophy is not known but could potentially involve an effect of sodium ions directly or due to sodium-dependent PKC activation.

Interestingly, the ability of both endothelin-1 and angiotensin II to activate NHE-1 appears to be impaired in the hypertrophic myocardium (7). Although the relevance of this finding is still uncertain, it is intriguing to suggest that this represents an adaptive protective mechanism to limit further NHE-1 dependent effects under conditions of already existing hypertrophy.

3. EXPERIMENTAL EVIDENCE FOR NHE-1 INVOLVEMENT IN HYPERTROPHY AND HEART FAILURE

3.1 Evidence In Cardiac Myocytes And Isolated Tissues

As just noted, NHE-1 represents a key downstream factor activated by

a variety of hypertrophic stimuli, a property which is likely not shared by any other cellular regulatory process. The ability of a large number of hypertrophic stimuli to activate NHE-1 does not necessarily confirm or prove cause and effect relationships. However, this concept has been reinforced in studies which have utilized pharmacological inhibitors of the antiporter.

A number of investigators have used cultured neonatal cardiac myocytes or isolated tissues to demonstrate NHE-1 involvement in hypertrophy. These in vitro approaches are advantageous in that one can study precisely the direct hypertrophic responses to relevant stimuli in the absence of other contributing factors. Moreover, the study of the cellular and molecular basis for hypertrophy is facilitated by these types of investigations. The primary limitation of using in vitro methodology is that the complete picture of the complex underlying mechanisms contributing to both the hypertrophy and the subsequent development of heart failure, cannot be fully addressed. In addition, the use of neonatal cells could be problematic and results using such cells should be interpreted cautiously since these cells likely possess different relative receptor subtypes or cell signalling mechanisms from adult tissues. Nonetheless, the use of cultured neonatal cardiac myocytes have provided useful and important information for understanding mechanisms of hypertrophic responses. Using these cells it has been shown that norepinephrine-induced protein synthesis in cultured rat cardiomyocytes can be blocked by NHE inhibitors (8). The above comments notwithstanding, adult cardiac myocytes have also been shown to exhibit NHE-1 dependent hypertrophy to agonists. Specifically, Schluter and coworkers demonstrated increased cellular RNA mass in adult rat ventricular myocytes following exposure to phenylephrine which was abolished by the NHE-1 inhibitor HOE 694 (9).

In addition to pharmacologically-induced hypertrophy, stretch-induced stimulation in protein synthesis in neonatal cardiac myocytes as well as stretch-induced alkalinization in feline papillary muscles can be blocked by NHE inhibitors (10,11). The exact nature of signalling mechanisms in stretch-induced hypertrophy are not known with certainty but may involve an NHE-1 dependent MAP kinase and Raf-1 activation (11). These investigators proposed that NHE-1 activation results in subsequent intracellular calcium elevation secondary to p sodium influx with calcium activating MAP kinase and Raf-1 (11). Interestingly, these authors showed that the effect of NHE-1 inhibition was restricted to stretch-induced effects with no effect against angiotensin II or endothelin-1 induced hypertrophy (11). The precise of paracrine or autocrine factors still needs to be determined in greater detail. However, one report demonstrated a potential link between stretch induced NHE-1 activation and distinct paracrine factors. In that study, the ability of stretch to induce intracellular alkalinization in cat papillary muscles was blocked both by NHE inhibition as well as

angiotensin AT_1 and endothelin ET_A antagonists (10). Although hypertrophy was not assessed in that study, the finding does link stretch-induced NHE-1 activation to a paracrine/autocrine-dependent process.

The above findings dealing with stretch induced changes is of potential clinical relevance as mechanical stress is an important initiating factor in the myocardial remodelling process after tissue injury. Thus, irrespective of the precise cellular mechanisms, there is strong evidence that initial stress could activate intracellular cascades leading to NHE-1 stimulation. What is still unclear at present how NHE-1 contributes to the remodelling and heart failure process. This is discussed in section 4 of this chapter.

3.2 Evidence In Animal Heart Failure Models

3.2.1 Postinfarction Heart Failure

Research into the potential role of NHE, or more specifically, NHE-1, in the development of heart failure have utilized in vivo approaches and well-defined heart failure models. Initial experiments in this area have utilized relatively nonspecific inhibitors of the antiporter such as amiloride. Indeed, it has been shown that orally-administered amiloride reduces fibre diameter in both the rat coronary ligation (12) and murine dilated cardiomyopathy models (13). The former is of particular usefulness in that it is characterized in a well-defined series of postinfarction adaptive responses culminating in heart failure similar to that seen in the clinical setting. We (2,14) and others (15) have utilized this model to identify potential beneficial effects of cariporide, the NHE-1 selective inhibitor, on both early and late postinfarction-induce heart failure. Orally administered cariporide completely abrogated the increased length of surviving myocytes after one week after coronary artery occlusion and improved contractile dysfunction (14). It is important to note that these effects occurred in the absence of afterload reduction. Moreover, improved hemodynamics was associated with an almost complete abrogation or left ventricular hypertrophy. We further expanded these studies to a more chronic (3 month) follow up period where both adaptive, ie hypertrophic, and heart failure responses are more pronounced. As with the one week study, cariporide significantly attenuated left ventricular dysfunction which included a marked attenuation of left ventricular end-diastolic pressures (2). This was associated with improved shortening of surviving myocytes (2). Because infarct size was unaffected, the results likely reflect the ability to decrease the hypertrophic/remodelling process by NHE-1 inhibition.

3.2.2 Right Ventricular Hypertrophy And Heart Failure

We have recently also observed that NHE-1 inhibition attenuates right ventricular hypertrophy in a model of pulmonary hypertension produced monocrotaline. In that study, monocrotaline produced severe pulmonary artery remodelling and intimal thickening resulting in compensatory right ventricular hypertrophy (16). Cariporide had no effect on vascular responses but significantly attenuated the right ventricular and improved hemodynamic parameters suggesting a direct antihypertrophic effect of NHE-1 inhibition in this model (16).

3.2.3 Genetic Animal Models Of Heart Failure

While the evidence for NHE-1 involvement in postinfarction myocardial remodelling and heart failure is emerging, it is also becoming apparent that the exchanger is also of importance in other heart failure scenarios. A particularly impressive observation was recently reported by Engelhardt and colleagues (17) who used transgenic mice which developed heart failure due to β1 receptor overexpression. In these animals, provision of dietary cariporide completed abrogated manifestations of heart failure including myocyte hypertrophy, diminished left ventricular function and interstitial fibrosis.

In unpublished studies, NHE-1 inhibition with EMD 87580 also markedly reduced necrosis, calcium and sodium overload and improved survival in cardiomyopathic hamsters (G Bkaily, personal communications)

NHE-1 has also been implicated in hypertension-induced myocardial hypertrophy and failure. For example, in the spontaneously hypertensive rat (SHR) myocardial hypertrophy was accompanied by increased NHE-1 activity (18). Interestingly, a number of classical antihypertensive agents including losartan, nifedipine as well as captopril were effective in reducing both the hypertrophy concomitantly with NHE-1 normalization. Furthermore, cariporide has been shown to reduce hypertrophy in the SHR which occurred independently of blood pressure reduction thus implicating NHE-1 as a direct hypertrophic factor in hypertension (19).

4. POTENTIAL MECHANISMS FOR NHE-1 INVOLVEMENT IN HYPERTROPHY AND HEART FAILURE

The precise mechanism for NHE-1 involvement in the hypertrophic response or heart failure remains be determined. It is unlikely to be related

to intracellular pH since other intracellular pH-regulatory processes would most likely be recruited to assure intracellular pH homeostasis. Thus, in view of the multiplicity of intracellular pH-regulatory mechanisms in the cardiac cell, it is doubtful that intracellular acidosis would be markedly greater in hearts treated with NHE-1 inhibitors. Indeed, a recent study dissociated the NHE-1 dependent hypertrophy in cultured rat ventricular myocytes exposed to either phenylephrine or isoproterenol from intracellular pH changes (20).

A potentially interesting hypothesis involves sodium ions which are important mediators of cell hypertrophy (21,22). Accordingly, the accompanying reduction in sodium entry occurring during NHE-1 inhibition may represent the major basis for salutary effects of NHE-1 inhibition on hypertrophy and heart failure. Indeed, in a recent study which utilized neonatal rat ventricular myocytes exposed to hypertrophic agents it was proposed that NHE-1-dependent sodium influx is a major contributor to hypertrophy produced by these factors including α_1 -adrenoceptor agonists, endothelin-1, or phorbol ester and which involved sodium-induced activation of PKC isoforms, especially PKCδ and PKCε (22). Indeed, inhibitors of PKC were found to reduce the hypertrophic response whereas the NHE-1 inhibitors HOE-694 decreased both the hypertrophy as well as PKC activation (22), thus reinforcing this link between PKC and NHE-1. However, other cell signalling mechanisms may also participate. For example, as already alluded to, stretch-induced cardiac cell hypertrophy was also associated with Raf-1 and MAP kinase activation, both of which were blocked by HOE-694 (16). Taken together, these findings are suggestive of NHE-1 involvement in the activation of various kinases resulting in cell growth.

5. CONCLUSION

Emerging evidence is suggestive of an important role of NHE-1 in cardiac hypertrophy and heart failure. As such, NHE-1 inhibitors could be attractive candidates either alone or as adjunctive therapy for the attenuation of maladaptive myocardial responses which culminate in heart failure. Substantial work needs to be done particularly in terms of understanding underlying the mechanism for NHE-1 involvement.

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Chapter 15

MECHANISMS UNDERLYING NHE-1 INVOLVEMENT IN MYOCARDIAL ISCHEMIC AND REPERFUSION INJURY

Morris Karmazyn
Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada

1. INTRODUCTION

Intracellular pH in the cardiac cell must be carefully regulated particularly since accumulation of intracellular protons can have profound effects on cardiac contractility. The NHE represents one of the key mechanisms for restoring pH_i following ischemia-induced acidosis by extruding protons concomitantly with Na influx in an electroneutral process. At present, 8 NHE isoforms have been identified (see Chapter 2) with the NHE-1 subtype representing the primary one found in the mammalian heart. When NHE-1 is activated, the simultaneous entry of Na during NHE activation likely represents an important route for increasing intracellular Na concentrations during various conditions. In the ischemic cell particularly, the activation of NHE by intracellular proton generation and the resultant entry of Na results in a potential disastrous consequence due to the fact that the excess Na cannot be extruded because of depressed Na-K ATPase activity. As a result, the reduction in the transmembrane Na gradient will result in increased intracellular Ca levels via the Na-Ca exchanger producing intracellular Ca overload and cell death. Pharmacological studies with NHE inhibitors, especially have extensively and repeatedly demonstrated protective effects in a large number of experimental models. Inhibition of NHE as a therapeutical tool has now entered the clinical arena as reflected by substantial effort by the pharmaceutical industry to develop potent NHE-1

specific inhibitors with potential as effective therapeutic agents in patients with coronary artery disease. Indeed, some of these agents have either undergone or are currently in the process of clinical evaluation. It is interesting that in addition to its potential role in mediating ischemic and reperfusion injury, NHE appears to also contribute to the postinfarction hypertrophic and remodelling process which can lead to the eventual development of heart failure. As such, a potential added benefit of NHE-1 inhibitors may include attenuation of the evolution of infarcted myocardium to failure. The aim of this review is to briefly summarize our current knowledge of NHE-1 in the heart in terms of its regulation, and of particular relevance, the importance of NHE-1 in cardiac ischemia. We will also discuss the pharmacology of NHE inhibitors and the development of novel and specific NHE-1 inhibitors for cardiac therapeutics. The pharmacology of NHE inhibitors can be readily separated into two periods of study and development, the first representing an era where amiloride or its analogues represented the primary pharmacological tools to probe the exchanger. These studies have provided important evidence as well as mechanisms for the role of NHE in cardiac injury (1). It is clear however that the therapeutic benefits of this research will likely materialize with more recently-developed NHE-1 specific inhibitors aimed at clinical development, of which two have already entered clinical trials.

2. CELLULAR LOCALIZATION OF NHE-1 IN THE HEART

Interestingly, immunohistochemical studies have revealed that NHE-1 is predominantly localized at the intercalated disk region of atrial and ventricular myocytes in close proximity to the gap junction protein, connexin 43, and to a lesser extent, along the transverse tubular system (2). Connexin 43 and the sarcoplasmic reticulum Ca release channel (i.e. ryanodine receptor) are highly pH_i-sensitive. Thus it has been speculated that due to its apparent localization, NHE-1 regulates the pH microenvironment of these pH_i-sensitive proteins, and thereby influences cell-to-cell ion dependent communication and intracellular Ca levels (2).

3. REGULATION OF NHE-1 ACTIVITY IN THE CARDIAC CELL

The major stimulus that regulates NHE-1 activity under normal physiological conditions is pH_i.(3). Within the normal physiological pH

range (pH 7.1-7.3), NHE-1 activity is negligible, but as pH_i decreases, the exchanger becomes rapidly activated. The reason for this rapid activation is due to the so-called H sensor, which is found on the cytoplasmic surface of the exchanger and accounts for the sensitivity of the exchanger to pH_i. Although the exact nature of the molecular mechanisms involved in activation by the H sensor is poorly understood, it is believed that binding of H to this site induces a conformational change of the NHE oligomer resulting in an increase in NHE activity (4). Extrinsic factors such as hormones, growth factors, cytokines, and autocrine/paracrine regulators modulate NHE-1 activity by increasing the sensitivity of the H sensor to pH_i, thus causing a shift of NHE-1 activity towards an alkaline range; that is, NHE-1 activity increases at a less acidic pH_i. This shift in pH_i dependence is accomplished mainly via phosphorylation reactions of the C-terminal domain of the exchanger, which is responsible for determining the pH_i set point value of the H sensor (5,6).

3.2 Activation of NHE-1 By Cardioactive Paracrine And Autocrine Factors

Although the most important factor for stimulating NHE-1 is intracellular proton generation, it is important to note that various factors important in cardiac pathology acting via signalling pathways can increase NHE-1 activity, including endothelin-1 (7,8), angiotensin II (9,10), α_1-adrenergic agonists (11,12), thrombin (13) and growth factors (6,14,15). The effects of these agonists generally involve phosphoinositide hydrolysis and activation of kinases resulting in NHE-1 activation (5, 15-17). In addition, cardiotoxic ischemic metabolites such as hydrogen peroxide (18) and lysophosphatidylcholine (19) have also been demonstrated to stimulate NHE-1 activity, a phenomenon which likely contribute to the cardiotoxic effects of these factors.

3.3 Role Of Phosphorylation

Structure-function studies have indicated that NHE-1 contains consensus sequences for mitogen-activated protein (MAP) kinases, which have been implicated in NHE-1 phosphorylation and activation (18, 20) and it has been established, using rabbit skeletal muscle, that MAP kinases can directly phosphorylate the C-terminal domain of NHE-1(20). A role for $p90^{rsk}$ in MAP-kinase dependent phosphorylation of NHE-1 has been demonstrated in rat myocardium (21). In addition, hypoxia, hypoxia with reoxygenation

(22) hydrogen peroxide (18) and other reactive oxygen species (23) have been known to stimulate the MAP-kinase signalling pathway, which can contribute to NHE-1 activation. In a recent study, it was shown that that MAP-kinase-dependent pathways including p90(rsk) and ERK1/2 are important in regulating NHE-1 and demonstrate a marked increase in activity toward NHE-1 during myocardial ischemia and reperfusion (24).

3.4 Phosphorylation-Independent Regulation

NHE-1 activity can also be regulated via phosphorylation-independent mechanisms (25,26). For example, deletion of the cytoplasmic C-terminal domain at residue 635 removes all phosphorylation sites although this reduces growth factor activation of NHE-1 by only 50% (20). In addition, NHE-1 activity can be completely eliminated following deletion of residues 567-635, while preserving mitogen-stimulated phosphorylation (26). These studies therefore strongly implicate factors other than phosphorylation which may be involved in NHE-1 activation. Bertrand et al (25) reported that the cytoplasmic C-terminal tail of NHE-1 contained two domains capable of binding calmodulin with either high (CaM-A residues 636-656) or low (CaM-B residues 567-635) affinity. The high affinity CaM-A site is believed to be important in transport regulation. Deletions of residues 636-656 render NHE-1 constitutively active, as if cytosolic Ca levels were continuously elevated. Based on these observations, it was suggested that at basal intracellular Ca levels, the unoccupied CaM-A binding domain exerts an autoinhibitory effect that is relieved upon Ca/calmodulin binding (26). Although this has yet to be demonstrated in the myocardium, it nevertheless suggests an alternative method for NHE-1 activation in pathological conditions in which intracellular Ca levels are elevated.

3.5 Role Of ATP

ATP has also been demonstrated to regulate NHE-1 activity. Depletion of cytoplasmic ATP results in reduced transport activity of the exchanger (27,28) although it appears that this is unlikely related to changes in the phosphorylation state of the exchanger (28). It has been hypothesized that a yet-to-be-identified ancillary protein may mediate the effect of ATP depletion on NHE-1 activity. It is believed that an ATP-dependent reversible association of a cofactor may regulate the exchanger, and that upon binding of ATP; this cofactor will dissociate from the exchanger and remove its inhibitory effect (28). Whether this has relevance to the

regulation of NHE-1 in the ischemic myocardium is not known, particularly since ATP depletion during ischemia is a relatively slow process. However, the possibility exists that very low levels of the nucleotide in ischemic myocytes could potentially counter the stimulatory effect of intracellular acidosis on NHE-1 activity.

3.6 Activation By G Proteins

It is also worth mentioning that a number of G proteins can modulate NHE-1 activity, although they have only been demonstrated to do so in noncardiac tissue. The mechanisms by which G-proteins stimulate NHE-1 activity are very complex, and vary depending on the G-protein type. For example, $G_{\alpha q}$ and $G_{\alpha 12}$ have been shown to regulate NHE-1 activity primarily via a PKC-dependent pathway, whereas $G_{\alpha 13}$ mediates NHE-1 activity via a PKC-independent pathway (29-31). $G_{\alpha 13}$ utilizes a distinct kinase cascade using the Rho family of GTPases (Cdc42 and RhoA) to activate NHE-1 through MAP/extracellular signal-regulated kinase kinase 1 (MEKK1)-dependent (Cdc42) and –independent (RhoA) pathways (32).

A phosphoprotein termed calcineurin homologous protein (CHP) has been reported to inhibit both serum- and GTPase-activated NHE-1 activity by binding to the regulatory domain of the carboxy terminus (33). It is not known at present what the role of CHP is regulating NHE-1 activity in the myocardium under normal or pathological conditions.

4. MECHANISMS UNDERLYING NHE INVOLVEMENT IN MYOCARDIAL ISCHEMIC AND REPERFUSION INJURY

A scheme for NHE-1 involvement for ischemic and reperfusion-induced injury is shown in Figure 1. The primary basis for NHE-1 involvement in acute injury reflects the inability to extrude sodium by the ischemic cardiac cell due to Na-K ATPase inhibition which occurs in concert with NHE-1, activation, the latter occurring as a consequence of increased proton generation during ischemia. Indeed, it could be stated that inhibition of Na-K ATPase is a *prerequisite* for NHE-1 involvement in ischemic and reperfusion injury and that in the absence of such inhibition NHE-1 activation would be unlikely to represent a deleterious influence on the myocardium. In addition, as noted above, NHE-1 is further activated by various hormonal, autocrine, or paracrine factors as well as metabolites produced either extracellularly or intracellularly during myocardial ischemia

including hydrogen peroxide and LPC. Thus, the net result is a multifactorial stimulation of NHE under pathological conditions, not due only to increased intracellular acidosis but also due to activation by external factors. Such marked NHE-1 stimulation increases an elevation in intracellular sodium levels which in turn increases intracellularcalciumlevels *via* Na-Ca exchange resulting in cell injury. Recent evidence suggests that the Na-Ca exchanger may actively contribute to calcium overload via reverse-mode calcium entry since transgenic mice overexpressing this exchanger show an increased sensitivity to ischemic injury, which would not be expected if elevated calcium would occur primarily via reduced efflux (34). It is interesting that this increased sensitivity was observed in male, but not female animals (34).

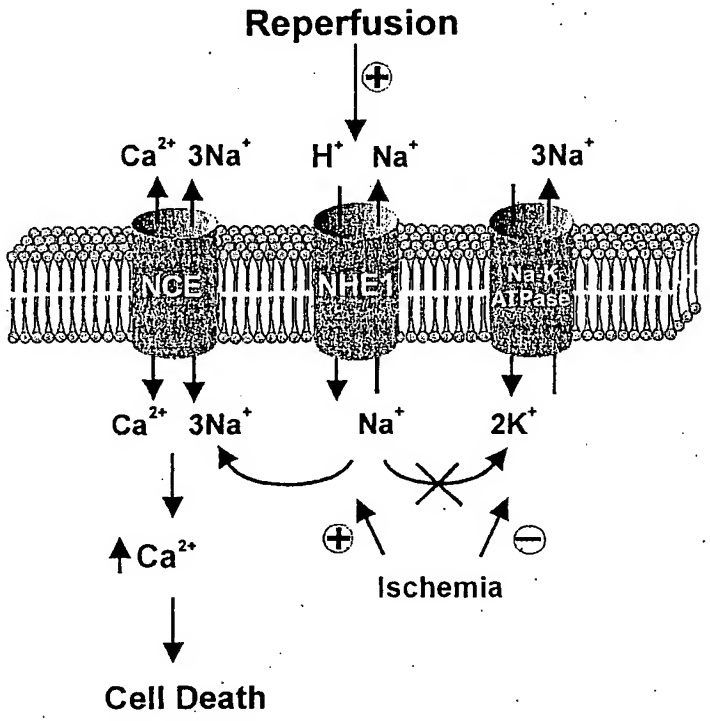

Figure 1. Schematic illustrating the role of NHE-1 in mediating myocardial ischemic and reperfusion injury. See text for discussion.

It should be noted that an alternate concept regarding a reperfusion-induced NHE-dependent injury through Ca-independent mechanisms has also been proposed, which has been termed the pH paradox. This hypothesis proposes that the reduction in intracellular ATP levels during myocardial ischemia results in phospholipase and protease activation which would normally produce cell membrane injury, however because these enzymes possess pH optima in the alkaline range their detrimental effects are attenuated by ischemia-induced acidosis. Upon reperfusion the rapid restoration of pH_i reverses the suppression of proteases and other enzymes seen during the ischemic period and results in cell death (35). In addition, the restoration of pH_i stimulates the formation of the mitochondrial membrane permeability transition which results in depression of ATP resynthesis via oxidative phosphorylation pathways (35). The relative contribution of this process to NHE-1-dependent cardiac injury is however not certain but is supported by studies utilizing individual myocytes illustrating a protective effect of NHE inhibition against reoxygenation which can be dissociated from intracellular Ca levels (36). It is possible that this mechanism may contribute specifically to reperfusion injury *per se* but obviously would not account for the potential role of NHE-1 inhibition during ischemia in the absence of reperfusion where the exchanger plays a critical role.

Acute stimulation of NHE-1 activity may not be the sole mechanism by which the NHE-1 system is upregulated under ischemic conditions. In this regard, ischemia as well cardiotoxic metabolites produced during myocardial ischemia can stimulate expression of NHE-1 suggesting that increased synthesis of the antiporter contributes to its upregulation under pathological conditions (37).

It has been suggested that mitochondrial ATP-sensitive K^+ channels play a role in the cardioprotective effects of NHE-1 inhibitors (38). In this regard, both the non-selective ATP-sensitive K^+ channel blocker glibenclamide as well as the mitochondrial ATP-sensitive K^+ channel blocker 5-hydroxydecanoate prevented the salutary effects of cariporide against ischemic and reperfusion injury in rabbits (38). Using isolated rat hearts, we have recently found that although 5-hydroxydecanoate prevented the preconditioning effect, it had no effect on the protective effects of cariporide (39). Thus, the exact role, if any, of ATP-sensitive K^+ channels in the cardioprotection afforded by NHE-1 inhibitors require further study and elucidation.

4.1. Myocardial Protection By NHE Inhibitors

The extensive documentation demonstrating cardioprotective effects of NHE inhibitors has strongly supported the concept of the antiporter's involvement in cardiac injury, especially under conditions of ischemia and reperfusion. The earlier studies utilized amiloride or amiloride analogues to demonstrated cardioprotective properties, however more recent data utilizing drugs targeted for clinical development reported excellent and consistent protection in a wide variety of experimental models and animal species which is likely unmatched in the cardioprotection literature. As recently reviewed (1,40) a number of such agents are either in clinical or preclinical development and all have been show to protect the myocardium against either ischemia or reperfusion injury or against the direct deleterious effects of cardiotoxic compounds produced by the ischemic myocardium. Moreover, there appear to be no discrepant results which fail to show protective effects of NHE-1 inhibitors. In addition, the protective effects of NHE-1 inhibitors appear to be species-independent. A particularly striking feature of NHE-1 inhibitors is their ability to protect against various forms of dysfunctions including reduced mortality, limitation of infarct size, improvement of functional recovery after reperfusion, reduction of arrhythmias, attenuation of calcium and sodium dyshomeostasis, reduction of apoptosis as well as preservation of metabolic status such as attenuation of high energy phosphate depletion.

4.2. Does NHE-1 Mediated Injury Occur During Ischemia Or Reperfusion, Or Both?

Many of the newer drugs have been tested for their ability to protect the myocardium when administered only at reperfusion, a property which would be important in terms of treatment of patients who present with acute myocardial infarction. Most agents do indeed demonstrate protective effects when administered at this period although it should be stated that, in general, such protection is less than that seen with pre-ischemia drug administration. From a mechanistic perspective, these findings are not surprising since NHE-1 activation during ischemia contributes substantially to the sodium and calcium overloading conditions and resultant cell injury with further NHE-1 activation occurring immediately upon reflow. As such, *optimum* protective effects of NHE-1 inhibitors are likely realized when treatment can be maintained during both ischemia and reperfusion.

The above comment notwithstanding there is evidence that NHE-1 inhibition only at reperfusion inhibits myocardial injury (41) and one group

has proposed that the primary locus for myocardial protection is inhibition of reperfusion induced NHE-1 activation (42). The latter scenario is questionable in view of studies demonstrating NHE-1 dependent sodium and calcium loading during ischemia preceding reperfusion (43,44). Instead, the concept that NHE-1 inhibitors offer optimum protection when administered only at reperfusion likely reflects the ability of these agents to protect only under certain experimental conditions, for example when hearts are paced at low rates which precludes sodium accumulation during the ischemic period (45). When all studies are taken together, the most logical conclusion is that maximum protection by NHE-1 inhibitors is attained with the drug present during both the ischemic and reperfusion periods, whereas some protection can still be attained with sufficiently high concentrations administered only at reperfusion. Indeed, this has been demonstrated in various studies employing both in vitro and in vivo approaches (1,40). For example, as shown in Figure 2, cariporide provides excellent protection in terms of infarct size reduction when administered prior to coronary artery ligation although a significant, albeit reduced protection is evident when given at reperfusion (46). The site of protection by NHE-1 inhibitors is of utmost importance in applying these findings to the clinical scenario. Accordingly, ideal salutary effects will likely be observed when the drug is present during the ischemic episode such as during coronary artery bypass surgery. Indeed, as discussed elsewhere in this volume, this is a major indication of NHE-1 inhibitors in current clinical trials

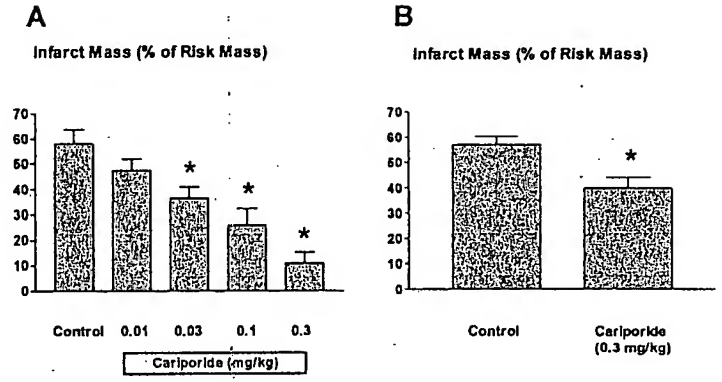

Figure 2. Dose-dependent cardioprotective effects of cariporide in terms of infarct size reduction when administered prior to coronary artery ligation in the rabbit (Pane A). Panel B demonstrates the diminished but still significant protection by the highest cariporide dose when administered 15 minutes before reperfusion. Data from ref (46) courtesy of Dr Wolfgang Linz, with permission.

5. POTENT ANTIARRHYTHMIC EFFECTS OF NHE-1 INHIBITION

Because NHE-1 is an electroneutral system it is difficult to difficult to appreciate how blocking the antiporter exerts antiarrhythmic properties. Yet, NHE-1 inhibitors have been extensively demonstrated to exert antiarrhythmic effects which appear to be superior to classical antiarrhythmic agents. A large number of studies have demonstrated antiarrhythmic properties of NHE-1 inhibitors. Although the mechanistic basis for antiarrhythmic properties of NHE-1 inhibitors is unknown, the effect may be secondary to inhibition of intracellular calcium and sodium levels as well as reduced tissue injury. Indeed, tissue protection and reduction of arrhythmogenesis likely share common NHE-1 dependent mechanisms reflecting enhanced intracellular sodium and calcium levels. As for the cardioprotectiive effects of NHE-1 in general, antiarrhythmic properties has also been shown in a large variety of experimental animal models.

There is unequivocal evidence that either orally or intravenously administered cariporide exerts impressive antiarrhythmic, and particularly antifibrillatory effects in rats subjected to coronary artery occlusion and reperfusion, Thus, cariporide completely prevented the incidence of ventricular fibrillation and significantly reduced both the incidence and duration of ventricular tachycardia extrasystolic activity (47,48). Antiarrhythmic properties of cariporide have also been in this model when the drug was administered as a bolus intravenous injection (49,50). These investigators also reported synergistic interaction in terms of antiarrhythmic effects with a combination of subthreshold cariporide and ischemic preconditioning (50).

Antiarrhythmic effects have also been shown with other NHE-1 inhibitors such as BIIB513, when administered intravenously prior to coronary artery ligation (51). BIIB 513 also reduced ischemia induced arrhythmias and reperfusion induced VF in dogs subjected to coronary occlusion and reperfusion(52). However, inhibition of ischemia-induced arrhythmias was restricted to those occurring after 10 minutes of occlusion with no efficacy on those arrhythmias occurring within the first 10 min ischemic period. Other specific NHE-1 inhibitors including SL 59,1227, a non-acylguanidine NHE-1 inhibitor and FR168888 have been shown to exert effective antiarrhythmic properties in a rat coronary artery occlusion-reperfusion (53,54). In the latter study, FR168888 was shown to be more effective than the class I antiarrhythmic agent lidocaine.

The mechanism(s) for the antiarrhythmic properties on NHE-1 inhibitors is not known. However, as NHE-1 is electroneutral it is likely that the

effects are secondary to tissue protection although, as noted below, antiarrhythmic effects can also be demonstrated in the absence of infarction. However, a relationship between antiarrhythmic properties of cariporide and its ability to reduce infarct size has been reported (50) although this does not necessarily confirm cause and effect relationship. An association between antiarrhythmic properties of cariporide and improved myocardial metabolic status has also been proposed (55). Cariporide has been shown to completely suppress the shortening of the monophasic action potential during early reperfusion of the ischemic porcine myocardium (56), a phenomenon which would potentially explain the antifibrillatory effect of NHE-1 inhibition in the reperfused myocardium. Indeed, cariporide had no effect on action potential shortening during ischemia ischemia per se (56).

As discussed in detail in Chapter 20, Gazmuri and colleagues have shown that NHE-1 inhibitors may also hold promise for post VF resuscitation. These investigators used a rat model of cardiac arrest produced by electrically-induced VF in which resuscitation was attempted with chest compression followed by electrical defibrillation (57). Seventy-five percent of rats treated with cariporide demonstrated spontaneous defibrillation following chest compression whereas 100% of control rats required electrical defibrillation. Moreover, the depth of chest compression was significantly less in cariporide-treated rats. This study is important not only because of its potential to identify a novel treatment for cardiac arrest, but also because it demonstrates that NHE-1 inhibition exerts beneficial effects against arrhythmias which are independent of myocardial infarction.

Evidence has also emerged that the effect of arrhythmogenic agents which initiate arrhythmias in the absence of ischemia or which exacerbate ischemia or reperfusion-induced arrhythmias can also be mitigated by NHE-1 inhibition. For example, arrhythmias produced by kappa-opioid receptor activation were shown to be blocked with amiloride analogues (58). Results with these compounds, however, should be interpreted somewhat cautiously in view of the non-specific nature of this group of drugs compared to newly-developed NHE-1 specific inhibitors. NHE-1 inhibition with HOE 694 has also been shown to attenuate the exacerbation of reperfusion arrhythmias produced by the α_1 adrenergic agonist phenylephrine (59).

Atrial arrhythmic activity may also be NHE-1 dependent. In this regards, it has recently been reported that cariporide blocks atrial electrophysiological changes (electrophysiological remodeling) in dogs produced either by rapid atrial pacing to simulate atrial fibrillation, or by occlusion of the right coronary artery (60).

6. OTHER POTENTIAL MECHANISMS MEDIATING CARDIOPROTECTIVE EFFECTS OF NHE-1 INHIBITORS

Although we have concentrated our discussion on the direct impact of NHE-1 inhibitors on the ischemic and reperfused myocardium, there is evidence that inhibiting NHE-1 may offer additional protective effects via alternate mechanisms. For example, NHE-1 inhibition reduces neutrophil activation and hence reduces the potential deleterious effects of neutrophil-derived mediators such as reactive oxygen species on the myocardium (61,62). Indeed, it appears that NHE-1 inhibitors have a direct protective effect against the deleterious effects of hydrogen peroxide on the normally perfused as well as ischemic reperfused myocardium (63,64).

Inhibition of NHE-1 is also associated with improved endothelium-dependent coronary vasodilation following ischemia and reperfusion (65,66) and reduced neutrophil adhesion to endothelium (67). Thus, it appears that NHE-1 inhibition offers endothelial protection. How this occurs is uncertain but it has recently been suggested that NHE-1 mediates cytokine-induced inflammatory response in endothelial cells (68). As well, it has been shown that NHE-1 inhibition attenuates the upregulation of the adhesion molecule ICAM-1 in coronary endothelial cells (69).

Recently, a new concept regarding cardioprotection by NHE-1 inhibition has been proposed which involves the inhibition of norepinephrine release from sympathetic nerve endings. Interestingly, this concept implicates presynaptic histamine H3 receptors whose activation results in NHE-1 inhibition. Thus, the H3 receptor agonist imetit was found to inhibit NHE-1 activity and diminish norepinephrine release (70). This finding suggests a further alternate target for NHE-1 inhibition in cardioprotection under in vivo conditions.

7. CONCLUSION

The past 15 years or so have seen substantial progress and advances with respect to the understanding of NHE in the heart particularly its role in mediating myocardial ischemic and reperfusion injury, and more recently, its potential role in long-term myocardial adaptation and development of heart failure. In terms of ischemic injury and cardiac protection these advances have lead, relatively rapidly, to the establishment of clinical trials aimed at determining whether selective NHE-1 inhibition protects high-risk patients with coronary artery disease or those with acute myocardial infarction. It is very likely and hopeful that new therapeutic strategies will emerge, based on both the clinical trials which have been or are currently being undertaken, as well as the obvious rapid development of a large number of new NHE-1 inhibitors. Nonetheless, much work needs to be done in this regard particularly in terms of understanding of the regulation of NHE-1 in chronic responses and how the system can be ideally modulated for therapeutic strategies, either alone or as adjunctive therapy with other treatment modalities, for the treatment of heart disease.

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Chapter 16

CHEMISTRY OF NHE INHIBITORS

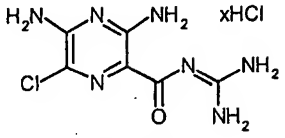
Hans Jochen Lang
Aventis Pharma Deutschland GmbH, Frankfurt am Main, Germany

1. INTRODUCTION

For more than a decade after the discovery of the Na^+/H^+ exchanger (NHE) in 1976, amiloride-type agents were the only tools available for the investigation of the consequences of NHE inhibition in biological systems. During this period, increasing knowledge about the role of NHE in pathology highlighted the need for novel NHE inhibitors for clinical development. A crucial step on the way to new classes of NHE inhibitors, which led to the discovery and development of cariporide (Hoe 642), was the realization of the importance of the guanidine group for NHE inhibitory activity. Hundreds of patents and papers have been published within the last decade, containing thousands of novel NHE-inhibitory compounds with different structures. This chapter revisits briefly the approach that led to the discovery of cariporide and describes a classification of NHE inhibitors based on their chemical structures.

1.1. The Importance Of The Guanidine Moiety

Following the first evidence for the existence of a cellular NHE provided by Murer et al. (1) and the seminal work of Lazdunski and coworkers on this system (2), amiloride (Figure 1) remained the only inhibitory tool for several years.



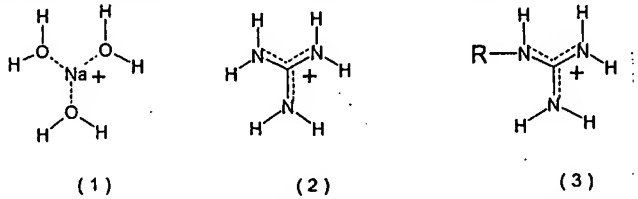
amiloride

Figure 1. The chemical structure of amiloride

In the 1980s, Vigne et al. (3) described the structure-activity relationship of several amiloride derivatives synthesized by Cragoe (4). Numerous amiloride derivatives had been synthesized by Merck researchers and by chemists at several other pharmaceutical companies in the 1960s and later, when the antikaliuretic, pseudo-aldosterone antagonistic properties of amiloride were recognized (4). This antikaliuretic activity of amiloride was found to occur due to inhibition of the epithelial sodium channel (ENaC). Although there was extensive variation in other substituents among these compounds, the pyrazine heterocycle of amiloride and its derivatives was considered to be essential for bioactivity and therefore remained unvaried. Thus, structure-activity optimizations were predominantly focused on the substituents of the heterocyclic pyrazine moiety. The synthesis of N-alkylated amiloride derivatives in position 5 of the heterocyclic pyrazine system led to the development of potent NHE inhibitors, such as DMA, HMA, EIPA, etc (see "Heteroaroylguanidines" below).

By the middle of the 1980s, my co-workers and I had begun looking for a completely new chemical approach to the synthesis of novel NHE inhibitors that differed from the pyrazine series, for the following two reasons: 1. The side effects of amiloride derivatives, reported in the literature, were likely to limit their therapeutic potential (5). 2. Further synthetic opportunities for new pyrazine substitution patterns seemed to be limited, due to the comprehensive synthetic work carried out by the Merck researchers and by other pharmaceutical companies. Thus, the probability of success for a breakthrough that might lead to the development of novel NHE inhibitors with significantly improved therapeutic potential seemed to be very low while the fundamental amiloride structure was retained.

A publication by Paolini from 1969 (6), rediscovered by Natochin (7), provided the molecular basis for our approach to new NHE inhibitors. Paolini had concluded that: 1. In aqueous medium, the Na^+ ion was triagonally surrounded by 3 molecules of water. 2. In terms of charge, shape and size, the tri-hydrated Na^+ ion (structure [1], Figure 2) was nearly identical to the guanidinium ion (structure [2], Figure 2).



(1) (2) (3)

Figure 2. Structures of the tri-hydrated Na^+ ion [1] and the native [2] or R-substituted [3] guanidinium ion.

The hypothesis of Natochin (7) was that the small guanidine portion, but not the pyrazine heterocycle, of the amiloride molecule was essential for biological activity, due to the binding of this portion to the Na^+ binding site of ENaC. Therefore, we chose to focus on the extracellular Na^+ binding site of NHE. The first goal was to find an organic molecule or a chemical group that binds to the extracellular Na^+ binding site of NHE, due to an ability to mimic exactly the Na^+ ion. In other words, the Na^+ binding site should not be able to distinguish the drug from the natural substrate, the Na^+ ion.

Other questions remained to be resolved: Was Natochin's hypothesis, which was established for ENaC blockers, also valid for NHE inhibitors? Would it be possible to lower the half maximum inhibitory concentration (IC_{50}) of guanidine (structure [2], Figure 2), estimated to be >1000 µM, to the ≤1 µM range that would be expected of a potential therapeutic agent? Approximately 100 derivatives of 10 different classes guanidines were synthesized by variations of R in structure [3] (Figure 2). Striking success was obtained by the synthesis of our first benzoylguanidines (structure [4], Figure 3), which were found to inhibit NHE1 activity with IC_{50} values in the ≤1 µM range. Of these compounds, Hoe 694 and cariporide (Figure 3) have since become standard pharmacological tools for subtype (NHE1) specific NHE inhibition. Moreover, cariporide was selected for clinical development. The story of the discovery of novel NHE inhibitors and cariporide has been described in more detail by Roald Hoffmann, winner of the 1981 Nobel Prize in Chemistry (8). The structural variability of the benzoylguanidine moiety (structure [4], Figure 3) opened the road of success for the synthesis of numerous new NHE inhibitors.

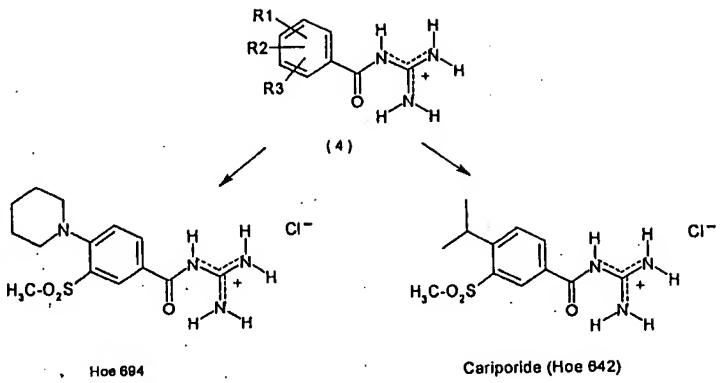


Figure 3. The basic benzoylguanidine structure [4] and two NHE inhibitors derived from this structure, Hoe 694 and cariporide (Hoe 642).

2. CHEMICAL CLASSES OF NHE INHIBITORS

As noted above, several thousand NHE inhibitors of different chemical structures have now been described, in hundreds of patents and publications. The objective of this section of the chapter is to propose a new chemical classification for present and future NHE inhibitors, which divides these agents into 5 convenient classes based on their structural features.

2.1. Aroylguanidines

NHE inhibitors of the aroylguanidine class are characterized by an aryl rest, such as phenyl, which is combined via a carbonyl group to the guanidine residue, as shown in structure [4] (Figure 3). Aroylguanidines represent the first class of NHE1 inhibitors with therapeutic potential. Cariporide mesilate (Figure 4) was the first NHE inhibitor selected for further clinical development by Aventis. Cariporide has been found to show not only high inhibitory potency and selectivity for NHE1 (IC_{50} for NHE1, NHE2 and NHE3 of 0.05, 3 and 1000 μM, respectively, in fibroblasts (9)), but also good aqueous solubility for parenteral application and excellent properties of resorption and bioavailability.

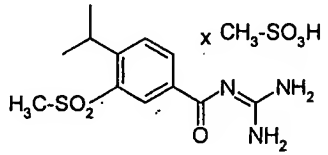
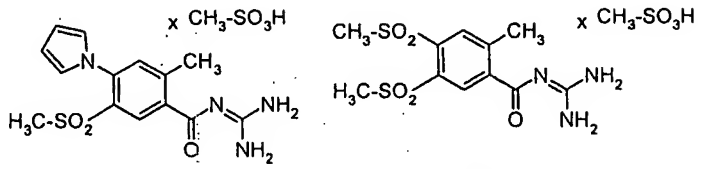


Figure 4. Structure of cariporide mesilate (Hoe 642).

Cariporide represents the "mother compound" of NHE inhibitors. Indeed, the suffix "poride" has been introduced into the World Health Organization approved International Nonproprietary Names (INNs) of subsequent NHE inhibitors.

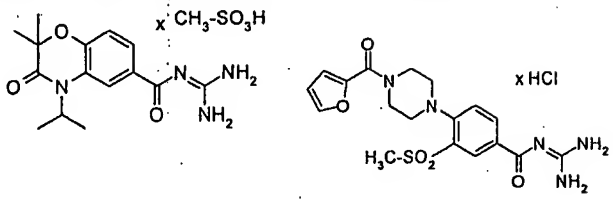
Within the aroylguanidine NHE inhibitor class, eniporide (10) and EMD 87580 (11) are under clinical development by E.Merck (Darmstadt, Germany). These compounds, whose structures are illustrated in Figure 5, are potent and selective NHE1 inhibitors. For example, eniporide exhibits IC_{50} values of 0.01, 0.27 and 700 μM for NHE1, NHE2 and NHE3, respectively, in mouse fibroblasts (10). A distinguishing feature amongst these two agents is that eniporide seems to be better suited for parenteral treatment and EMD 87580 for oral applications.



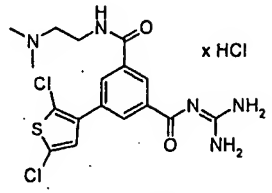
eniporide EMD 87580

Figure 5. Structure of eniporide (EMD 96785) and EMD 87580.

Other aroylguanidines selected for evaluation are KB-R9032 from Kanebo (12,13), BIIB 513 from Boehringer Ingelheim (14) and FR-183998 from Fujisawa (15). The estimated IC_{50} values for these agents, whose structures are illustrated in Figure 6, are 24 nM for KB-R9032 in rat ventricular myoctes (13), 29 nM for BIIB 513 in HT29 human gut cancer cells (14) and 0.3 nM for FR-183998 in rat lymhocytes (15).



KB-R9032 BIIB 513



KB-R9032

Figure 6. Structures of KB-R9032, BIIB 513 and FR-183998.

2.2. Heteroaroylguanidines

Heteroaroylguanidines are characterized by a heteroaromatic ring system, which is combined with the guanidine residue via a carbonyl group. They may be further subdivided into 2 subclasses, as below.

2.2.1. 6-Membered Heteroaroylguanidines

The general structure of NHE inhibitors within this subclass is illustrated in Figure 7.

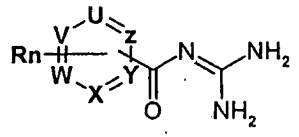


Figure 7. Basic structure of 6-membered heteroaroylguanidines.

Amiloride (Figure 1) and its derivatives (Figure 8), characterized by a pyrazine heterocycle, represent the first type of NHE inhibitors and belong to this subclass. They have been widely used as scientific tools, although they display limited NHE subtype selectivity. Furthermore, while Karmazyn (16) and others have shown these agents to possess cardioprotective properties, none is currently under clinical development as an NHE1 inhibitor due to undesired side effects (5).

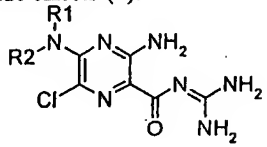


Figure 8. Basic structure of amiloride derivatives developed by Merck (4). In 5-N-dimethylamiloride (DMA), R1 and R2 are methyl; in 5-(N-methyl-N-isobutyl)-amiloride (MIA), R1 is methyl and R2 is isobutyl; in 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), R1 is ethyl and R2 is isopropyl.

Other heteroaroylguanidines with pyridine and quinoline heterocyclic nucleus, such as TY-12533 from Toa Eiyo (17) and MS-31-038 from Mitsui Toatsu (32) (Figure 9), have also been reported to be selected for development.

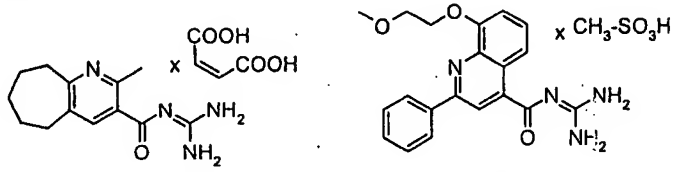


Figure 9. Structures of TY-12533 and MS-31-038

2.2.2. 5-Membered Heteroaroylguanidines

The general structure of NHE inhibitors within this subclass is illustrated in Figure 10.

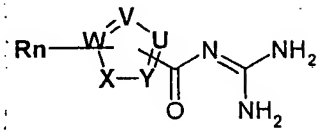


Figure 10. Basic structure of 5-membered heteroaroylguanidines.

More recently, several NHE1 inhibitors with a five-ring heterocyclic core have been reported to possess considerable therapeutic potential. These pyrrazoloylguanidines (Pfizer) were found to be highly potent NHE1 inhibitors with remarkable selectivity for NHE1 over NHE2. Zoniporide (Figure 11), which has been selected for further development, exhibits IC_{50} values of 0.06, 120 and 700 μM for NHE1, NHE2 and NHE3, respectively, in fibroblasts transfected to express these NHE subtypes (18).

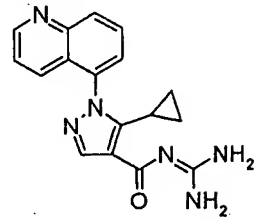


Figure 11. Structure of zoniporide.

Indoloylguanidines represent another type of 5-membered heteroaroylguanidines. Researchers of Sumitomo Pharmaceuticals have characterized several indoloylguanidines (Figure 12) as very potent NHE1 inhibitors with therapeutic potential in combating ischemia/reperfusion induced injury in the heart and the CNS. Of these agents, SM-20220 has been shown to exhibit IC_{50} values for NHE1 of 5 nM in cultured neurons and 20 nM in glial cells (19), with a corresponding value for SMP-300 of 6 nM in rat myocytes (20).

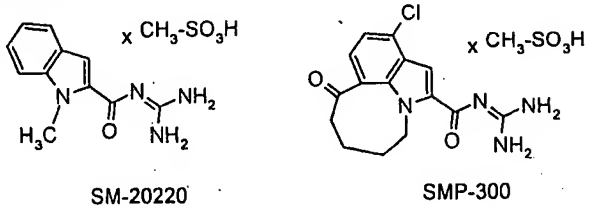


Figure 12. Structures of SM-20220 and SMP-300.

2.3. Spacer-Stretched Aroylguanidines

Spacer-stretched aroylguanidines are characterized by an aryl rest which is combined with the carbonylguanidine residue via a spacer moiety "A-B" and a carbonyl group (Figure 13).

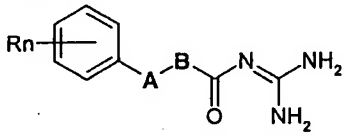


Figure 13. Basic structure of spacer-stretched aroylguanidines.

Spacer-stretched aroylguanidines, which have a spacer group "A-B" of two atoms (such as an ethylene group -C=C-) between the aryl nucleus and the carbonylguanidine group, have been shown to be active NHE inhibitors. Some patent applications of cinnamoylguanidines (structure 5, Figure 14) were filed (21). Structural variations have led to further cinnamoyl-like NHE1 inhibitors within the last few years. For example, NHE inhibitory cinnamoylguanidines were obtained if R1 and R2 of the structure formed a ring system, resulting in indenoylguanidines (structure 6, Figure 14) (22) or the benzocycloheptene derivatives of Fujisawa (structure 7, Figure 14) (23). Interestingly, Sumitomo's indoloyl guanidines (Figure 12) combine two elements of this classification; they can be considered as 5-membered heteroaroylguanidines (see above) as well as cinnamoyl-like guanidines.

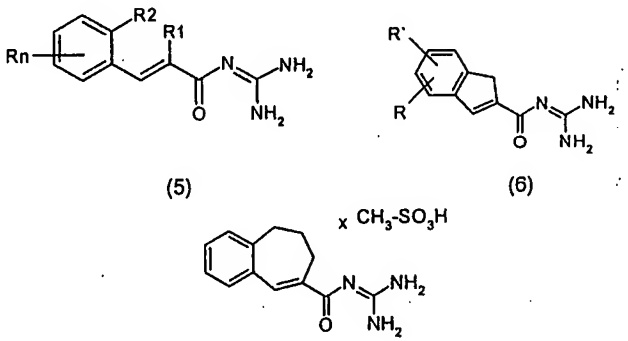


Figure 14. Structures of cinnamoylguanidines.

The close relationship between ethylene and cyclopane groups is a well known chemical and pharmaceutical phenomenon. Thus, replacement of ethylene in cinnamoylguanidines (structure 5, Figure 14) by a cyclopropane

bridge leads to highly potent NHE1 inhibitors (structure 8, Figure 15), as recently shown by researchers at Bristol-Myers Squibb (24). Arylcyclopropanecarboxyl guanidines represent another class of promising spacer-stretched aroylguanidines. Of these compounds, BMS-284640 (Figure 15) was selected for further development, because of its potency and remarkable selectivity for NHE1 over NHE2. Thus, BMS-284640 has been shown to exhibit IC_{50} values of 0.09, 1.8, >30 and 3.36 μM for NHE1, NHE2, NHE3 and NHE5, respectively, in AP1 cells transfected to express the relevant NHE isoforms (24).

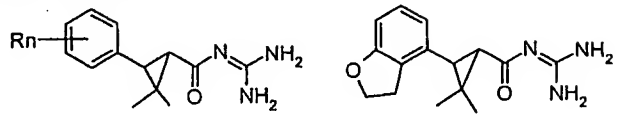


Figure 15. Structures of arylcyclopropanecarboxyl guanidines [8] and BMS-284640

The research effort that resulted in the different classes of NHE1 inhibitors described above was directed mainly towards an increased NHE1-inhibitory potency and/or selectivity. Nevertheless, by variations of substituents in the cinnamoylguanidine structure (Figure 14), a relative increase in NHE3-inhibitory potency has been observed. Thus, a structure-dependent shift from NHE1 inhibitors to inhibitors of other NHE subtypes appears feasible. Indeed, a special substitution pattern of the aromatic ring by Schwark and Heinelt (Aventis Pharma Deutschland GmbH) has resulted in potent NHE3 inhibitors (21,25). For example, S10519 (Figure 16) was found to have an IC_{50} of 70 nM for NHE3, in a mouse fibroblast cell line transfected to express this subtype.

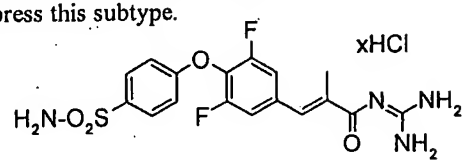
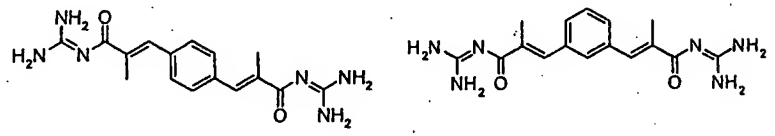


Figure 16. Structure of S10519.

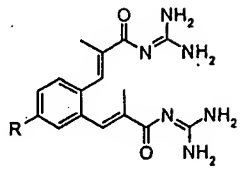
A surprising structure-activity relationship was observed in cinnamoylguanidines substituted by another methacryloyl-guanidine residue, –CH=C(Me)-CO-N=C(NH_2)$_2$ (25). Phenylene nuclei of S2121 and S2120 (Figure 17), which are substituted by identical methacryloyl residues in the 1,4- (para-) or the 1,3- (meta-) position, were found to be potent NHE1 inhibitors. Thus, the IC_{50} values against NHE1, NHE2 and NHE3 were 0.03, 0.81 and 1.13 μM, respectively, for S2121, and 0.002, 0.07 and 1.32 μM, respectively, for S2120, in mouse fibroblast cell lines expressing the relevant

NHE subtypes. In contrast, the 1,2-(ortho-) isomer S1611 showed predominantly NHE3 inhibitory activity. Thus, the corresponding IC_{50} values for the 1,2–bis(methacryloyl) derivatives were 4.70, 89 and 0.69 µM, respectively, for S1611 and 3.55, 80 and 0.23 µM, respectively, for S3226. S1611 and S3226 (Figure 17) are now used as physiological tools to study the consequences of NHE3 inhibition in epithelial tissues and in the CNS.



S2121

S2120

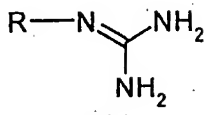


S1611/S3226

Figure 17. Structures of S2121, S2120, S1611 and S3226. R is H in S1611 and CH_3 in S3226.

2.4. Non-Acyl Guanidines

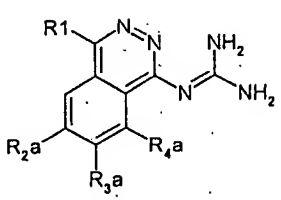
Non-acyl guanidines are characterized by a 5- or 6-membered (hetero)aromatic ring system, which is combined with the guanidine residue without a carbonyl bridge (Figure 18).



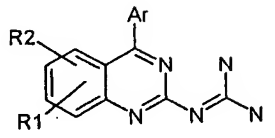
(11)

Figure 18. Basic structure of non-acyl guanidines.

In 1999, Watanabe et al. (Takeda Chemical Industries) reported about NHE-inhibitory cinnoline derivatives of structure [9] (Figure 19) containing no carbonyl link (26). Related quinazolinyl-guanidines (structure [10], Figure 19), claimed to display NHE3-inhibitory activities (no pharmacolocigal data), were described by Gericke et al. (E.Merck) in 2002 (27).



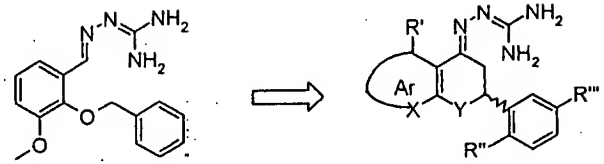
(9)



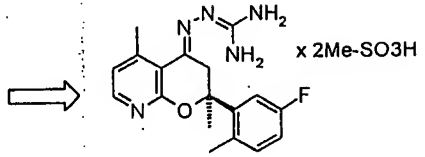
(10)

Figure 19. Non-acyl guanidine structures [9] and [10].

A promising series of NHE1 inhibitors with a cyclic quinolinylidene aminoguanidine structure (structure [11], Figure 20) have been obtained by Fukumoto et al. at Takeda (28). The scheme illustrated in Figure 20 suggests that the spacer stretching principle (see above) can also be applied to non-acyl guanidines, by the introduction of C=N (an ethylene-like spacer group) between the guanidine group and the heteroaromatic pyridyl system. T-162559 (Figure 20), which has an IC_{50} of 14 nM in rat platelets, was selected for development by Takeda (28).



(14)



T-162559

Figure 20. Structures of cyclic quinolinylidene aminoguanidine [11] and T-162559

2.5. Non-Guanidine NHE Inhibitors

Only a few publications have reported about NHE inhibitors without a guanidine residue. SL 59.1227, a new NHE inhibitor representing a novel class of imidazolylpiperidine compounds, was described by Lorrain et al. Sanofi-Synthelabo) (29). SL 59.1227, whose structure is illustrated in Figure 21, exhibited marked NHE1-inhibitory potency as well as

considerable selectivity for this subtype over NHE2 (IC_{50} values of 3.3 nM for NHE1 and 2.3 µM for NHE2) (29).

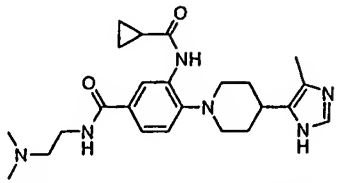


Figure 21. Structure of SL 59.1227

The norbornylamine derivative S11599 (Figure 22), which exhibits significant NHE3- inhibitory activity (IC_{50} of 0.5 µM in fibroblasts expressing NHE3) was selected for further evaluation at Aventis (30).

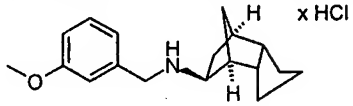


Figure 22. Structure of S11599.

Another family of non-guanidine NHE inhibitors comprises the natural polyamine squalamine (Figure 23) and its synthetic derivatives, which inhibit NHE3 (but not NHE1 or NHE2), possibly through an indirect mechanism via an intracellular pathway (31).

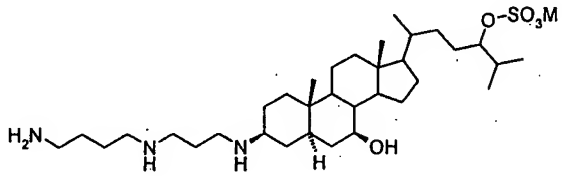


Figure 23. Structure of squalamine.

In closing, it is clear that four out of the five structural classes of NHE inhibitors described above are guanidine derivatives. This illustrates the impact that an old hypothesis can have in opening up new perspectives in medicinal chemistry.

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Chapter 17

DEVELOPMENT OF NHE INHIBITORS FOR CARDIOVASCULAR THERAPEUTICS

Wolfgang Scholz and Norbert Beier
Merck KGaA, Darmstadt, Germany

1. INTRODUCTION

Preclinical development of NHE subtype 1 (NHE-1) inhibitors dates back to the late 1980's. While amiloride had been characterized as a weak and non-specific inhibitor of NHE-1, several amiloride derivatives had turned out somewhat more specific and potent. However, due to low in vivo tolerability, their use was mostly limited to being applied as research tools. These limitations were overcome with the emergence of a new class of inhibitors based on a benzoyl guanidine structure, as HOE 694 (1988), cariporide (1992), eniporide (1994) and later EMD 87580 in parallel with several other compounds from different pharmaceutical companies (1).

Preclinical pharmacology of NHE-1 inhibitors as well as a growing understanding of cellular ion homeostasis pointed to a central role of NHE-1 in the pathophysiology of myocardial ischemia and reperfusion. NHE-1, being excessively activated by intracellular acidosis, was identified as a major contributor to Na+ and Ca++ overload of the endangered heart cell under these conditions. While fluorescence-, NMR- and histological findings pointed to a reduction of intracellular Na+ and Ca++ overload by NHE-1 inhibition, cardio-protective effects of NHE-1 inhibitors were demonstrated in vitro and in vivo in a multitude of different models and species. These findings included consistent and impressive reductions of infarct sizes in settings that allowed pretreatment, some reduction of infarct size with treatment on reperfusion, a prevention of ischemic and reperfusion arrhythmia, stunning and more permanent dysfunction.

Shaped by this kind of preclinical findings, the consequent clinical development of NHE inhibitors was initially driven by the pathophysiologic implications of the role of NHE-1 as pH regulating protein in the ischemic/reperfused cardiomyocyte. The insight into the involvement of NHE-1 in the detrimental disturbance of intracellular sodium and calcium in myocardial ischemia/reperfusion as well as the experimental evidence accumulated in numerous preclinical trials in different species (2), led to some first approaches to test these concepts under clinical conditions.

2. CLINICAL TRIALS PERFORMED WITH NHE-1 INHIBITORS IN PATIENTS EXPOSED TO MYOCARDIAL ISCHEMIA/REPERFUSION

After having established safety in healthy volunteers and patients with coronary heart disease, in 1995, the NHE-1 inhibitor cariporide was administered to patients undergoing coronary artery bypass grafting (CABG) and valve replacement. In this tiny pilot trial (n=20), treated patients showed a tendency towards a reduced need for positive inotropic agents in the first days after surgery (3). While being much to small to demonstrate statistical significant differences between groups, this first clinical trial offered an ideal setting for the use of NHE-1 inhibitors in ischemia/reperfusion. Since surgery has to adhere to a certain time frame, patients can be treated before the onset of ischemia, during ischemia and during reperfusion. These were exactly the conditions under which the most convincing and consistent effects had been observed in preclinical studies (4).

Nevertheless, in 1996 a second pilot trial (n=100) was set up with cariporide in a different indication, including patients with acute (anterior) myocardial infarction (AMI), which were undergoing reperfusion therapy by coronary dilatation (PTCA) (5). Of course, under these conditions, the NHE inhibitor could only be given prior to reperfusion in patients who were already suffering from myocardial ischemia when included into the trial. While there was some preclinical evidence for this approach (6), results from animal models were not as unanimous as seen with treatment prior to ischemia (7-10), and probably also more dependent from specific compound characteristics and the use of relatively high dosages (7). In any case, the trial in which about 50% of the patient population was eligible for final evaluation (two successful ventriculographies required), showed a trend towards an improvement of functional parameters as well as a significant reduction of enzyme release in treated patients (5).

2.1 The GUARDIAN Trial

Based on the impression that the scope of clinical applications of NHE-1 inhibitors as cardioprotective agents might be relatively broad, these activities were followed by a huge combined phase II/III trial with cariporide, which included three different patient populations (n=11,800 altogether). In this (GUARDIAN) Trial, the NHE-1 inhibitor was given at different doses to patients either suffering from unstable angina/non Q-wave MI, or undergoing high risk PTCA, or being subjected to high risk bypass surgery. Primary endpoints were the occurrence of MI and mortality within 28 days.

At first evaluation, the results of the GUARDIAN trial were considered as quite disappointing. In the overall study population, GUARDIAN showed a risk reduction for primary endpoints of about 10% in the highest dose group only, an effect, which did not reach significance (11). Altogether, the study failed to demonstrate a reduction of MI and mortality in this mixed population of patients, which had all for some time and under diverse circumstances suffered from acute myocardial ischemia. At first view, this lack of efficacy seemed to stand in stark contrast to the preclinical data seen with NHE-1 inhibitors.

A closer look at the three indications included in GUARDIAN revealed a more complex picture. Efficacy turned out quite different in different indications. Primary endpoints were significantly reduced (-25%) in CABG patients treated with the highest dose of cariporide. While no such significant reduction was seen in patients undergoing PTCA, or in patients suffering from unstable angina, there was a significant shift from apparently bigger myocardial infarctions (Q-Wave MI) to smaller infarctions (non-Q-Wave MI) in these populations. Of course, this effect was not reflected in the primary endpoint, which was the total number of myocardial infarctions, thus being quite insensitive to a possible change of the severity of MIs in the study.

2.2 The ESCAMI Trial

While the recruitment of patients into GUARDIAN was well under way, another clinical trial (ESCAMI) was started testing the very potent NHE-1 inhibitor eniporide in patients with AMI undergoing acute PTCA or thrombolysis, an indication, which had been excluded from GUARDIAN. Eniporide has shown consistent preclinical evidence in animal models of coronary ligation and reperfusion when given prior to reperfusion (12). Primary endpoint in this phase II dose finding trial (n=1,400) was enzyme release during the first 72 hrs after reperfusion. An interim analysis, which

was performed for further dose selection after the first 400 patients had been analyzed during the trial (n=80-90 per group), indicated a significant reduction of enzyme release in one dose group and a trend in a second one. Surprisingly, this finding was not confirmed at the end of the trial, when a group size of about 400 patients had been reached. Treatment with the NHE-1 inhibitor failed to cause a significant reduction of enzyme release at any dose used.

In view of the previous cariporide trial, in patients with acute MI undergoing PTCA, which had given some evidence of efficacy of a NHE-1 inhibitor in such a setting and in view of the ESCAMI interim analysis, such results was certainly unexpected. Nevertheless the negative results derived from a group size of ~400 could (and can) not be denied.

2.3 The EXPEDITION Trial

In 2001, a more thorough evaluation of the GUARDIAN results and especially the effects seen in the cardiac surgery patients, had led to the initiation of another large phase III trial (EXPEDITION) (13), where the NHE-1 inhibitor cariporide was given to patients undergoing high risk CABG. Primary endpoints were the occurrence of MI or death one week and 6 months after surgery. The trial had been planned to include about 7000 patients dependent on the observed event rate. In fall of 2002, the Drug Safety Monitoring Board, for reasons unreleased to the public stopped the recruitment of EXPEDITION (Aventis Press Release, July 24, 2002; www.aventis.com). At that time about 5,700 patients had already been included in the trial. The final outcome might be known in Spring 2003,after the publication of this volume, when the 6 months data of the patients will be available.

3. APPARENT CONTRADICTION BETWEEN PRECLINICAL DATA ON NHE-1 INHIBITORS AND THE OUTCOME OF CLINICAL TRIALS

For preclinical as well as clinical researchers, it seems important to better understand the apparent contradictions between preclinical data and clinical outcome, or may be the failure to translate preclinical models and their results into meaningful clinical settings especially in the development of NHE-1 inhibitors. While a more thorough discussion of the matter might not fit to the scope of this chapter, a few remarks seem to be suitable in this context.

As mentioned before, the best and most consistent cardioprotective effects of NHE-1 inhibitors were seen in preclinical models including a limited period of myocardial ischemia followed by reperfusion, where the compound was given before the ischemic insult and was present during ischemia and reperfusion. This was found and unanimously confirmed in numerous preclinical studies in different species. Cardioprotection was also observed in several studies when compounds were given later during the ischemic period, a few minutes prior to reperfusion (7, 8; see also table I in ref. 2)). However, these effects were less consistent, required higher dosage and were more species and compound dependent. No decrease of infarct size was seen in models of permanent coronary ligation where reperfusion of the ischemic myocardium was prevented by design (14, 15, 16).

Taken together, these findings seem to indicate that the main effect of such compound might be to slow down the process of degradation of the ischemic myocardium for a limited period of time (as illustrated below, Fig.1). An additional effect seems to be a reduction of injury during the last minutes of ischemia and during reperfusion, if such compound would reach the ischemic myocardium in sufficient concentrations shortly before reperfusion.

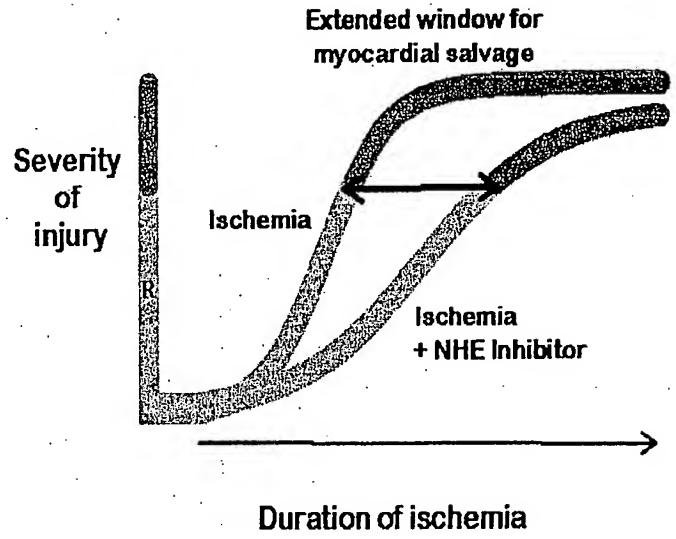

Figure 1. Time window for retardation of myocardial injury by NHE-1 inhibitors. Reproduced from ref (17) with permission.

To fully exploit the main effect as described above, it would be required that the NHE inhibitor has to be present in the endangered myocardium from the very beginning of ischemia – and during reperfusion, and that reperfusion would have to happen within a certain time window, within which a slow down of myocardial degradation was still relevant.

A closer look at the three different patient populations included in GUARDIAN reveals that this profile was met by only one of them, namely the patients undergoing cardiac surgery. Out of different reasons the fit of the other two populations to this profile seems quite questionable. In unstable angina, the myocardium is insulted by somewhat unpredictable sequences of varying durations of ischemia and reperfusion, which might or might not lead to myocardial infarction. In case of non-Q-MIs, it is clinical practice that patients do not receive any reperfusion therapy at all.

In PTCA, the ischemic periods caused by repeated inflation of the intra-coronary balloon are much too short to cause myocardial necrosis. Only if things go severely wrong, occlusion of a coronary vessel will occur, or debris from the process might cause some downstream ischemia. In both cases it seems questionable whether a window for reperfusion could be matched, which might allow for the cardioprotective effects of a NHE-1 inhibitor to become evident.

In case of the acute MI-reperfusion trials like ESCAMI, the apparent discrepancies between preclinical data and clinical outcome point to two different questions: 1) was reperfusion achieved timely enough to still permit the salvageof significant amounts of myocardial tissue? and 2) were the doses administered in patients high enough?

While there is always some gap between tightly controlled preclinical studies and clinical settings it might just have been the case that the specific conditions of preclinical models in which this kind of pre-reperfusion therapy worked, were not matched closely enough in the patient. One important difference was the varying and prolonged periods of ischemia (up to 6 hours between onset of symptoms and therapy), which the patients' myocardium had to suffer. Another discrepancy might be based on the dependence of effective doses on the animal species used in preclinical reperfusion studies. While relatively low doses of NHE-1 inhibitors were effective in dogs (8), much higher dosage was required in pigs (7), a fact, which complicated the estimation of a possible effective dose range in humans It seems therefore, that the apparent contradictions between preclinical ischemia/reperfusion studies with NHE-1 inhibitors and the clinical data could most likely be explained by some clear mismatch (as in two indications included in GUARDIAN) or some effective gaps between the exact conditions of preclinical models and the specific conditions and treatments the different patient populations were subjected to.

4. PRECLINICAL EVIDENCE FOR A CENTRAL ROLE OF NHE-1 IN POST MI REMODELING AND HEART FAILURE

While the further fate of NHE-1 inhibitors in indications related to acute myocardial ischemia/reperfusion might be very much dependent from the final results of the EXPEDITION trial, some years ago, the preclinical evaluation of NHE-1 and inhibitors has turned into a different direction.

When selective and well-tolerated NHE-1 inhibitors were found in the early 90ies, preclinical pharmacology was very much focused on the striking effects of such compounds in ischemia/reperfusion experiments. A few years later, the ongoing basic research revealed some quite complex mechanisms of activation and regulation this pH dependent ion transporter.

As integral part of the cells' pH regulation system, NHE-1 is activated by a decrease of intracellular pH (pHi). The set point of this effect is such that NHE-1 activity is negligible at physiologic pHi (~7.2). Under these conditions pHi is mainly regulated by bicarbonate dependent systems (18). However, the set point of NHE-1 activity can be shifted towards higher pHi values by a considerable number of effectors including thrombin and many neuro-hormones, including catecholamines, angiotensin II, endothelin-1 (19), aldosterone (20) and others (2). The presence of such hormones results in a significant activity of NHE-1 even at physiologic pHi.

As a consequence of such regulation, permanently increased levels of these neuro-hormones will also lead to permanently increased activity of NHE-1. A disease, where this kind of neuro-hormonal deregulation is found, is chronic heart failure, where maladaptive myocardial remodeling and fibrosis are induced. It seems remarkable that all such hormones involved in heart failure, which induce cardiac hypertrophy or fibrosis, are also known as activators of NHE-1. Indeed increased activity and expression of NHE-1 has been found in heart failure models (21) and in cardio-myocytes from patients with heart failure (22).

Since 1998, NHE-1 specific inhibitors have been investigated in preclinical studies of post MI remodeling and heart failure. Up to now, cariporide and EMD 87580 have shown striking effects in post MI rats as well as in different genetic animal models. This included a prevention of hypertrophy, fibrosis and heart failure in ß1-adrenergic receptor transgenic mice (23), prevention of myocardial degradation and marked reduction of mortality in cardiomyopathic hamsters (Bkaily, G unpublished observations), a prevention and even reversal of remodeling of cardiac myocytes, a prevention of cardiac functional degradation and a drastic reduction of mortality in post MI rats (Discussed in Chapter 14). These effects are possibly stronger than the benefits, which can be expected from ACE inhibitors at therapeutic doses and they are seen in the absence of any

cardiovascular side effects. Detailed discussion of the potential role of NHE-1 inhibitors in heart failure can be found in Chapter 14 of this volume.

5. CONCLUSIONS AND FUTURE DIRECTIONS

In the field of acute myocardial ischemia/reperfusion NHE-1 inhibitors have been investigated in clinical phase II/III trials in the indications unstable angina/non Q-wave MI, high-risk PTCA, acute MI/ reperfusion and high-risk cardiac surgery. While a significant risk reduction for clinical endpoints was observed in cardiac surgery, clinical studies in the other indications were negative. In view of the considerable size of the patient populations involved in these trials, it seems unlikely that the negative results will be challenged by further clinical investigations. The promising outcome in cardiac surgery will have to be confirmed by an additional phase III trial in this indication (EXPEDITION), which is currently under way. If successful, the EXPEDITION study could pave the way for the use of NHE-1 inhibitors in high risk CABG patients. The use of NHE-1 inhibitors in cardiac surgery is further discussed by Myers in Chapter 19 of this volume.

For heart failure as a clinical indication for NHE-1 inhibitors, some quite impressive preclinical background has been established. The effects seen in preclinical models are consistent from the cellular level over functional parameters to mortality. They are also consistently seen with different compounds in multiple studies and in different genetic models as well as in ischemia induced heart failure. Indeed, the rat coronary ligation model has been quite predictive for the clinical development of ACE inhibitors.

The present preclinical data of NHE-1 inhibitors in heart failure appear to be robust and to make a good case to start clinical development this indication. Possible endpoints for first small clinical trials could be derived from preclinical studies, where a marked reduction of cardiac natriuretic peptides has been observed (25). In the last few years, such peptides like BNP have been found to correlate tightly with the severity of the disease and with successful therapeutic intervention.

Therefore, further clinical development of NHE-1 inhibitors seems promising and also feasible in heart failure, an indication where the therapeutic need is still extremely high.

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Chapter 18

CARDIAC PROTECTION BY NHE INHIBITORS
Comparison With Other Cardioprotective Strategies

Garrett J. Gross
Department of Pharmacology and Toxicology, Medical College of Wisconsin, Watertown Plank Road, Milwaukee, WI, USA

1. INTRODUCTION

The sodium/hydrogen antiport is the major cardiac sarcolemmal membrane transporter responsible for the control of intracellular pH under normal physiological conditions and is thought to be activated and play a major role in the control of intracellular Na^+ and Ca^{++} concentrations during ischemia and/or reperfusion (1). In this regard, Karmazyn (2) in 1988 was the first investigator to demonstrate that amiloride, a potassium-sparing diuretic which also inhibits NHE activity in the heart, resulted in a marked improvement in the recovery of global contractile function in the isolated perfused Langendorff rat heart subjected to an ischemia-reperfusion protocol. These findings resulted in a surge of interest in developing inhibitors of NHE for use as cardioprotective agents. Initially, most compounds developed and tested were 5-amino substituted pyrazinoyl guanidine derivatives structurally related to amiloride, which were all found to be markedly cardioprotective. However, it was subsequently found that these compounds were not selective blockers of NHE alone and had other properties such as direct sodium channel blocking effects and cardiodepressant properties (3). Subsequently, chemists at Hoechst (3) and E. Merck (4) synthesized a new class of more selective inhibitors of the NHE-1 isoform, the benzoylguanidine derivatives, of which HOE-642 or cariporide, is the prototype. All of these compounds have been subsequently shown to be cardioprotective in all animal species and experimental models studied.

2. NHE-1 INHIBITION AND CARDIOPROTECTION

Since the original findings of Karmazyn demonstrating the cardioprotective effects of amiloride (2), many papers have been published which uniformly demonstrate the remarkable anti-ischemic efficacy of inhibiting NHE-1 in the myocardium. Therefore, a summary of the results obtained from many laboratories supporting a cardioprotective role for NHE-1 inhibitors and a comparison to that of ischemic and pharmacological preconditioning (IPC, PPC) will be the primary focus of this chapter.

2.1 Mechanisms Responsible For Cardioprotection

Prior to discussing the evidence for a cardioprotective effect of NHE-1 inhibition, it is essential to understand the pathophysiological basis and mechanisms responsible for the use and efficacy of these agents in alleviating the deleterious effects of ischemia and reperfusion in the heart. During myocardial ischemia the inside of the cell becomes acidotic which results in the activation of the NHE. This effect results in the extrusion of H^+ in exchange for Na^+. Since ischemia results in the inhibition of the Na^+-K^+ ATPase, which would normally extrude the excess Na^+, intracellular Na^+ accumulates. The increase in intracellular Na^+ leads to an accumulation of Ca^{++} since the Na^+ gradient normally present in the nonischemic heart is reduced and results in a decreased activity or reversal of the Na^+/Ca^{++} exchanger. Thus, during ischemia an increase in intracellular Ca^{++} would be expected to occur which would result in cell death due to activation of proteases and the rapid onset of rigor contracture unless timely reperfusion were to occur. However, during reperfusion further damage might also be expected as a result of the rapid washout of extracellular H^+ and a further enhancement of NHE activity to extrude the accumulated intracellular H^+. This would lead to a rapid increase in intracellular Na^+ and Ca^{++} and perhaps further damage to the myocardium. Based upon this sequence of events, it is not surprising that inhibitors of NHE-1 possess such a potent cardioprotective efficacy. However, whether this sequelae of events actually occurs is open to debate based on experimental evidence obtained in several in vitro isolated heart and myocyte studies where investigators have not been able to demonstrate increases in Na^+ or Ca^{++} during simulated ischemia or in globally ischemic isolated rat hearts (5,6). On the other hand, recent results from our laboratory addressed this question in isolated guinea pig hearts where we were able to directly determine cellular concentrations of Na^+ and Ca^{++} by the use of fluorescent dyes in the intact beating heart during global ischemia and following reperfusion in the presence and absence of an NHE-1 inhibitor, BIIB-513 (7). In these experiments, hearts were subjected to 30 minutes of ischemia and 70 minutes of reperfusion in

the absence or presence of 10 uM of BIIB-513 administered either 10 minutes prior to ischemia or immediately before reperfusion. The results clearly demonstrate that inhibiting NHE-1 before ischemia reduces Na^+ loading during ischemia and reperfusion and concomitantly reduces systolic Ca^{++} loading during reperfusion (Figure 1).

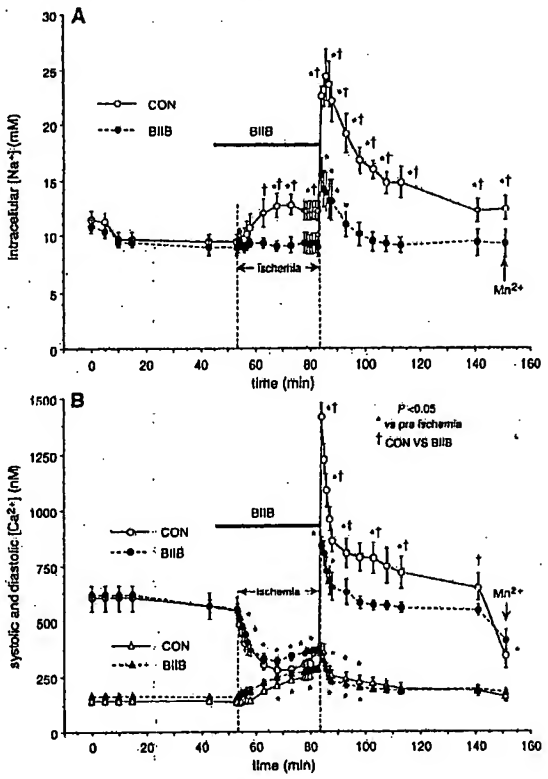

Figure 1. A. Intracellular Na^+ concentration ([Na^+]), and *B.* systolic and diastolic [Ca^{++}], before, during and after 30 min of global ischemia in the absence (CON) and presence of 10 uM BIIB-513 infused 10 min before ischemia and washed out on reperfusion. [Na^+] rose during ischemia in CON but not after BIIB-513, peak [Na^+] on reperfusion rose less after BIIB-513 than after CON and [Na^+] returned to preishemia levels only after BIIB-513. Peak systolic [Ca^{++}] rose less after BIIB-513 than in CON; systolic and diastolic [Ca^{++}] decreased during reperfusion, but systolic [Ca^{++}] returned to preischemic levels only after BIIB-513; and diastolic [Ca^{++}] returned to preischemic values during reperfusion after CON and BIIB-513. $MnCl_2$ was used to quench cytosolic [Ca^{++}]. *P<0.05 vs. preischemia; t<0.05 vs. BIIB. Reprinted from An et al. (7) by permission of the American Physiological Society.

These beneficial effects on Na^+ and Ca^{++} were accompanied by a marked reduction in myocardial stunning and infarct size and enhanced mechanical and metabolic function. Furthermore, inhibition of NHE-1 just

prior to reperfusion and during the initial 10 minutes of reperfusion also resulted in a reduction in Ca^{++} loading and an improved functional recovery of the heart. Similarly, in isolated rat hearts, Stromer et al. (8) using NMR spectroscopy, found that pretreatment with cariporide enhanced postischemic contractile function following 30 minutes of ischemia and 30 minutes of reflow by reducing Ca^{++} overload at end ischemia and during early reperfusion. Furthermore, these investigators showed that cariporide prolonged postischemic acidosis. These results obtained in the intact beating guinea pig and rat heart strongly support the hypothesis that cytosolic Na^+ and Ca^{++} are comarkers of ischemia reperfusion injury and that NHE and probably a decreased or reverse mode Na^+/Ca^{++} exchanger (NCE) are primarily responsible for the increase in Na^+ which occurs during ischemia and reperfusion and the increase in Ca^{++} during reperfusion. Thus, inhibition of NHE-1 would be expected to prevent these deleterious effects and result in a reduction in cell swelling, rigor contracture, cell necrosis, apoptosis and cardiac arrhythmias as will be summarized in the following sections of this chapter.

2.2 NHE-1 Inhibition And Infarct Size Reduction

The results of the first studies using the selective NHE-1 benzoylguanidine inhibitor, HOE-694, were reported by Klein et al. (9) and Rohmann et al. (10) in anesthetized pigs. Rohmann et al. (10) administered HOE-694 15 minutes before coronary artery occlusion or 15 minutes prior to reperfusion. In these studies, HOE-694 produced a marked reduction in infarct size following both modes of administration; however, the drug was more effective when given prior to the ischemic period. In accordance with these results, Klein and co-workers (9) also administered HOE-694 to pigs either 10 minutes prior to occlusion or 10 minutes prior to reperfusion and noted that the drug was also more effective when given as a pretreatment than when it was given just prior to reperfusion. These investigators also found that HOE-694 pretreatment resulted in an improvement in regional contractile function (myocardial stunning) 24 hours after drug administration. Similarly, in rat hearts, Wu et al. (11) demonstrated that BIIB 513, a new benzoylguanidine derivative, produced an 80-90% reduction in infarct size when administered 10 minutes prior to coronary occlusion and by 47% when given just prior to reperfusion. In a similar study in rabbits, Yamada et al. (12) found that the NHE-1 inhibitor, SM-20550, produced a dose-related reduction in infarct size of 30-70% when administered prior to occlusion and a 20-40% reduction when administered during ischemia. Based on a subsequent study in which infarct size was measured following 45, 70 and 90 minutes of ischemia in the absence or presence of pretreatment with HOE-642, Klein et al. (13) suggested that

NHE-1 inhibition increased the time window of protection or tolerance to irreversible ischemia by 20-25 minutes in the pig heart. In a more recent study (14) these same investigators found that infarct size in porcine hearts was reduced by an equal amount by cariporide when it was only administered during the first 30 minutes of a 45 minute ischemic period and during 45 minutes of ischemia and the first 10 minutes of reperfusion. These results led these investigators to conclude that cariporide does not exert any beneficial effect to reduce infarct size during the early reperfusion period. Using cariporide (HOE-642), Garcia-Dorado et al. (15) demonstrated that pretreatment with this compound in pigs reduced the onset of ischemic contracture, infarct size and the incidence of reperfusion arrhythmias; however, when cariporide was given just prior to reperfusion it did not reduce infarct size but still produced an antiarrhythmic effect. In agreement, Bugge et al. (16) demonstrated that ethylisopropylamiloride (EIPA) reduced infarct size in rabbits following preischemic treatment but not when EIPA was administered during the initial reperfusion period. Finally, Knight and co-workers (17) demonstrated that the newly developed NHE-1 inhibitor, zoniporide, produced a marked and concentration dependent reduction in infarct size in isolated and in vivo rabbit hearts when administered prior to coronary occlusion. No studies were performed when the drug was administered just prior to reperfusion.

Based on these studies, the results suggest that NHE-1 inhibition prior to ischemia is much more efficacious than when the drug is administered just prior to or at reperfusion. However, most of these studies were performed in rats, rabbits and pigs, species which are all known to possess a very sparse collateral blood flow. Thus, a minimal amount of drug would be expected to reach the ischemic area when administered just prior to or at reperfusion and the infarct size reduction would be minimal. Based upon this hypothesis, we undertook several studies in dogs (18), a species known to possess a more abundant collateral circulation during coronary artery occlusion. When either of 2 doses of the benzoylguanidine derivative, EMD 85131, was administered 15 minutes prior to occlusion the drug produced a significant dose-related reduction in infarct size (Figure 2). When the same 2 doses were administered 15 minutes prior to reperfusion, infarct size was still significantly reduced although to a lesser extent than when EMD 85131 was used as a pretreatment (Figure 3). Similarly, Ito et al. (19) demonstrated that another NHE-1 inhibitor SM-20550, significantly reduced infarct size when it was administered prior to occlusion or just prior to reperfusion in dogs. These authors further demonstrated that SM-20550 also alleviated damage to the microvasculature normally found after reperfusion in this model. Taken together, a summary of all studies performed in various species suggests that pretreatment with NHE-1 inhibitors uniformly produces a marked reduction in infarct size; however, the results obtained when administering these drugs just prior to reperfusion suggest that the

efficacy of NHE-1 is less or without effect particularly when given to animals with a sparse coronary collateral blood flow most likely due to a lack of sufficient amount of drug delivery to the area at risk during the ischemic period. In dogs, and most likely humans, with a well-developed collateral circulation due to long-standing coronary artery disease, NHE-1 inhibitors would still be likely to produce some reduction in infarct size when administered prior to reperfusion.

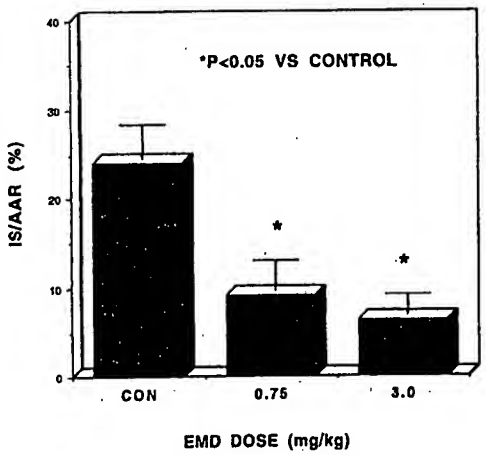

Figure 2. Effect of 2 doses of EMD-85131 (0.75 and 3.0 mg/kg IV) on myocardial infarct size expressed as percent of the area at risk (IS/AAR) in dogs. Drug was administered 15 min prior to a 60-min occlusion of the left anterior descending (LAD) coronary artery. All values are the mean±SEM (n=7-8 dogs/group). *P<0.05 vs. (CON) group. Reprinted from Gumina et al. (18) by permission of the American Society of Pharmacology and Experimental Therapeutics.

2.3 Comparative Effects Of NHE-1 Inhibition And Ischemic Preconditioning To Reduce Infarct Size

Ischemic preconditioning (IPC) is a phenomenon in which single or multiple brief periods of coronary artery occlusion result in a marked resistance to a subsequent more prolonged ischemic period (20). IPC, similar to NHE-1 inhibition, has been shown to reduce infarct size in all species studied and has been used as the gold standard against which all other cardioprotective interventions are compared. During the triggering

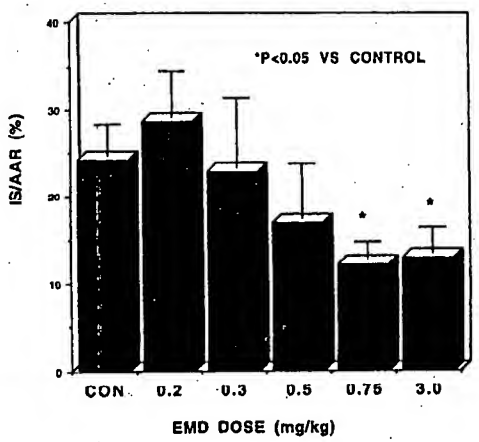

Figure 3. Effect of 5 doses of EMD-85131 (0.2. 0.3, 0.5, 0.75 and 3.0 mg/kg iv) on myocardial infarct size expressed as a percent of the area at risk (IS/AAR) in dogs. Drug was administered 15 min prior to reperfusion after a 60-min LAD occlusion. All values are the mean± SEM (n = 7/8 dogs/group). *P<0.05 vs. the (CON) group. Reprinted from Gumina et al. (18) by permission of the American Society of Pharmacology and Experimental Therapeutics.

phase of IPC in rats, it has been demonstrated that intracellular pH decreases and intracellular sodium increases (21). That preconditioned hearts recover from acidosis more rapidly than non-preconditioned hearts implies that NHE-1 might be activated by IPC (21). In contrast, Xiao and Allen (22) presented evidence which suggested that IPC slows the recovery of pH_i following ischemia in isolated perfused rat hearts which supports the hypothesis that IPC inhibits NHE-1 during early reperfusion and that this effect reduces Na^+ entry and Ca^{++} overload upon reperfusion. These seemingly contrasting results obtained in rats raise several important questions. Do NHE-1 inhibition and IPC protect the heart by similar mechanisms? Which intervention is more efficacious in limiting infarct size? What is the result of combining these 2 cardioprotective mechanisms, no effect, additive, synergism or antagonism?

To address these questions, Shipolini et al. (23) studied the potential interaction between NHE-1 inhibition and IPC by the use of cariporide in isolated rat hearts subjected to 40 or 60 minutes of ischemia and reperfusion. In hearts subjected to only 40 minutes of ischemia, both interventions produced nearly equivalent effects to enhance the recovery of left ventricular developed pressure (LVDP); however, when cariporide and IPC were combined there was a slightly greater recovery of LVDP. When the 2 interventions were used separately in the 60-minute ischemia model, again they both produced nearly equivalent effects to enhance the recovery

of LVDP; however, when the 2 interventions were combined an additive effect was observed. Based upon these results, these investigators concluded that the effects of NHE-1 inhibition and IPC were cardioprotective via distinct mechanisms and that the co-administration of an NHE-1 inhibitor to IPC may enhance the effect of IPC when the ischemic period was prolonged. In contrast, Munch-Ellingson et al. (24) and Sato et al. (25) demonstrated that NHE-1 inhibition and IPC produced nearly equivalent effects to reduce infarct size; however, NHE-1 inhibition did not produce a greater effect when combined with IPC. Based on these indirect results, these investigators proposed that NHE-1 inhibition and IPC were acting to protect the myocardium via different mechanisms. In agreement, Aye et al. (26) found in intact rat hearts that either IPC or cariporide were equally effective at reducing infarct size and the incidence of cardiac arrhythmias. They also demonstrated that cariporide and a subthreshold period of IPC had an additive effect in this model, which suggested to them that these 2 interventions did not share a common mechanism. In 2 other relevant studies, Miura et al. (27) and Gumina et al. (28) showed that the protective effects of NHE-1 inhibition to reduce infarct size in rabbits or dogs, respectively, were not blocked by either a protein kinase C (PKC) inhibitor (polymixin B), an adenosine receptor antagonist (PD 115199) or a K_{ATP} channel inhibitor (glibenclamide). In a similar vein, Hale and Kloner (29) found that the cardioprotective effect of cariporide to reduce infarct size in rabbit hearts was not reduced by pretreatment with 5-HD, a mitochondrial K_{ATP} channel blocker, although Miura et al.(30) found the opposite effect in rabbits. Since adenosine, PKC and the sarcolemmal or mitochondrial K_{ATP} channel have all been shown to be important triggers or mediators of IPC, these data strongly suggest that IPC and NHE-1 inhibition do not act via a similar signal transduction pathway.

Finally, in a study from our laboratory, Gumina et al. (31) compared the efficacy of NHE-1 inhibition and IPC to reduce infarct size in dog hearts subjected to either 60 or 90 minutes of coronary artery occlusion and 3 hours of reperfusion and to determine if any interaction exists between these 2 cardioprotective pathways. BIIB-513 was used as the NHE-1 inhibitor, and its effects were compared to those of IPC produced by one 5-minute occlusion or four 5-minute occlusions separated by 5 minutes of reperfusion, prior to the index ischemia of 60 or 90 minutes. Both BIIB-513 and IPC produced nearly equivalent reductions in infarct size in the 60-minute occlusion model; however, only BIIB-513 produced a significant reduction in infarct size in the 90 minute occlusion model when compared to a single or a four cycle model of IPC (Figure 4). When a dose of BIIB-513 that did not produce a significant reduction in infarct size alone was combined with one cycle of IPC in the 90 minute occlusion model, a greater than additive effect was observed. These results clearly suggest that NHE-1 inhibition has a greater ceiling of protection than IPC to reduce infarct size

in dogs subjected to a prolonged period of ischemia and that the combination of the 2 interventions may produce an additive or synergistic effect. In this regard, recent studies in our laboratory in dogs subjected to 120 minutes of coronary artery occlusion followed by 3 hours of reperfusion still demonstrated that BIIB-513 was capable of limiting infarct size whereas 4 cycles of IPC had no protective effect (data not shown). Neither intervention reduced infarct size when the occlusion period was extended to 3 hours. These results agree with previous work reported by our laboratory and by others which suggested that NHE-1 inhibition and IPC do not share a common mechanism. In this regard, Gan et al. (32) indicated that NHE-1 mRNA was markedly increased in response to ischemia, hydrogen peroxide and lysophosphatidyl choline in isolated rat hearts, however, in preconditioned hearts the expression of NHE-1 was markedly reduced. These data suggest that NHE-1 inhibition and IPC may be acting together to produce cardioprotection.

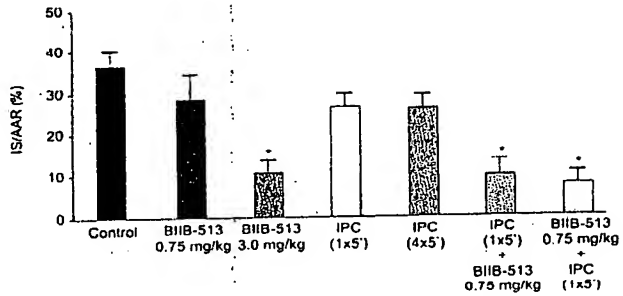

Figure 4. Effects of NHE-1 inhibition and IPC on infarct size following 90 minutes of LAD occlusion. BIIB-513 (0.75 or 3.0 mg/kg) was administered 15 min prior to occlusion or before IPC (1x5) or (4x5) was conducted. Infarct size was expressed as a percent of the area (IS/AAR). All values are the mean ± SEM (n=6-16 dogs/group). *P< 0.05 vs. the control group. Reprinted by permission of Drugs of the Future (Gross and Gumina, 26: 253-260, 2001).

2.4 Apoptosis And NHE-1 Inhibition

Considerable evidence has recently been shown to suggest that apoptosis or programmed cell death is another mechanism which may lead to myocardial injury following ischemia and reperfusion. The effect of NHE-1 inhibition by cariporide on apoptosis has recently been addressed by Chakraburti et al. (33) in the isolated rat heart subjected to selected periods of ischemia and 30 minutes of reperfusion. Evidence of apoptosis first was evident at 10 minutes of ischemia and was maximal at 30 minutes of ischemia. Pretreatment with cariporide 15 minutes before ischemia resulted

in a marked reduction in the number of cells exhibiting apoptosis (from 31± 3 to 2±1) and resulted in an enhanced recovery of contractile function following reperfusion. These results are exciting and suggest another mechanism by which NHE-1 inhibition may reduce myocardial injury and more studies are warranted to further examine mechanisms by which NHE-1 inhibitors alleviate apoptosis.

2.5 Antifibrillatory And Antiarrhythmic Effects Of NHE-1 Inhibitors

The results of several studies (26,34) have demonstrated that inhibition of NHE-1 results in a potent antifibrillatory and antiarrhythmic effect in dogs and rats. Aye et al. (26) showed that cariporide significantly reduced the incidence of ventricular fibrillation in dog hearts without any effects on the incidence of ventricular premature beats or ventricular tachycardia. Similarly, Gumina et al. (34) demonstrated that NHE-1 inhibition resulted in a reduction in the incidence of ventricular fibrillation in dog hearts at reperfusion and additionally reduced the incidence of arrhythmias at 20-30 minutes following a coronary artery occlusion in the canine heart. In rat hearts, Aye et al. (26) showed that cariporide produced a dose dependent reduction in the incidence and duration of ventricular tachycardia during ischemia and reperfusion and decreased the incidence of ventricular fibrillation upon reperfusion. Cariporide also decreased the arrhythmias produced by the administration of ouabain in rats. Whether these antiarrhythmic and antifibrillatory effects are a result of a direct electrophysiological effect of NHE-1 inhibition in the myocardium or the indirect result of the reduction in the severity of ischemia produced by their cardioprotective effects is unknown and is deserving of further investigation.

3. CLINICAL TRIALS WITH NHE-1 INHIBITORS: INFARCT SIZE REDUCTION

The efficacy of cariporide to exert an anti-ischemic effect in patients in a large multicenter clinical trial (GUARDIAN Trial) in 12,000 patients was recently completed and the results analyzed (35). The patients that were enrolled in this study were a heterogenous group who had unstable angina, a non-Q wave myocardial infarction or were patients undergoing coronary artery bypass graft surgery (CABG). Although cariporide was well tolerated by the patients, its overall effect to reduce indices of ischemic injury was not statistically significant when compared to a control group. However,

when a subset of patients who had undergone CABG surgery was further evaluated a significant beneficial effect was uncovered in the presence of a high dose of cariporide. In agreement, Rupprecht et al. (36) demonstrated that a dose of cariporide administered prior to reperfusion in patients undergoing percutaneous transluminal coronary angioplasty (PTCA) resulted in an improvement in functional and metabolic indices of ischemia 21 days after the PTCA procedure. In contrast, recent results in the ESCAMI trial showed that an NHE-1 inhibitor, eniporide, had no cardioprotective effect in patients undergoing reperfusion therapy for an evolving myocardial infarction (37). These results are encouraging and suggest that NHE-1 inhibitors may be an effective new means for treating patients with a myocardial infarction or as therapy for elective surgeries such as CABG or PTCA. However, based on the results of the ESCAMI trial, more clinical trials are necessary before any definitive conclusions can be made about the efficacy of these drugs as cardioprotective agents in man (37).

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Chapter 19

NHE-1 INHIBITORS:POTENTIAL APPLICATION IN CARDIAC SURGERY

M Lee Myers
Division of Cardiovascular Surgery, London Health Sciences Centre-Victoria and the University of Western Ontario, London, Ontario, Canada

1. INTRODUCTION

A variety of protective strategies are utilized in cardiac surgery to minimize procedure-induced ischemia/reperfusion injury and secondary myocardial dysfunction. This is of particular importance in acute ischemic syndromes where myocardial protection must also encompass resuscitation of the acutely ischemic or infarcting myocardium. Optimal myocardial protection is also of great importance in complex procedures where there is a need for prolonged aortic cross clamping, in the presence of left ventricular hypertrophy or dilatation as is commonly seen with valvular heart disease, or when extensive coronary disease results in inhomogeneous perfusion. Although there is currently increased interest in coronary revascularization without the use of cardiopulmonary bypass, the majority of cardiac procedures continue to be done with bypass, aortic cross clamping and cardioplegic arrest.

A great deal of scientific study has been undertaken to assess various formulations and modes of administration for cardioplegic solutions (1). Such solutions may be crystalloid or blood-based and may be administered at a range of temperatures. A large number of laboratory and clinical studies comparing the efficacy of crystalloid and blood cardioplegia have generally demonstrated advantages with blood cardioplegic solutions (2), although crystalloid solutions continue to be used in many centres with comparable clinical success. The use of systemic and myocardial hypothermia to

maintain cardiac arrest and decrease myocardial metabolic demand was the foundation upon which myocardial preservation techniques were developed. More recent clinical studies have generally demonstrated excellent myocardial preservation with warm blood cardioplegia (3) and warm or "tepid" cardioplegic solutions are now frequently utilized with minimal systemic cooling in routine cases.

Cardioplegic solutions can be administered antegrade into the aortic root, directly into the coronary ostia, or through saphenous vein grafts sequentially as distal anastomoses are completed. Maldistribution of cardioplegic solutions at the cellular level occurs, however, in the presence of occluded vessels with poor collateral and where use of arterial grafts precludes direct graft perfusion. The use of cardioplegia administered retrograde via the coronary sinus is an additional approach developed to permit more uniform cardioplegia distribution.

The ability to control not only the temperature, timing and mode of administration but also the specific formulation of the cardioplegic solution provides the surgeon with a unique opportunity for a direct pharmacologic approach to cardioprotection. A large body of experimental evidence has demonstrated that inhibition of sodium-hydrogen exchange is protective in the setting of myocardial ischemia/reperfusion, and the ability to inhibit the exchanger pharmacologically is a promising new approach to current myocardial preservation techniques.

2. MYOCARDIAL DYSFUNCTION AFTER CARDIAC SURGERY

Myocardial injury in association with cardiac surgery may result in reversible (i.e. myocardial stunning) or irreversible (i.e. myocardial necrosis) damage . Myocardial stunning refers to contractile dysfunction following reperfusion despite the absence of myocardial cellular necrosis. A range of associated reversible abnormalities have been demonstrated in experimental studies including cellular swelling, impaired calcium homoeostasis, increased capillary permeability, impaired endothelial/microvascular function and ATP depletion (4). Studies suggest that the production of toxic oxygen metabolites (5), cellular calcium overload (6) and cardiac troponin I proteolysis (7) are important etiologic factors.

Such reversible dysfunction is known to occur in patients undergoing cardiac surgery. For example, Fremes et al (8) demonstrated a significant improvement in left ventricular function between 6 and 24 hours postoperatively in coronary bypass patients managed with cold crystalloid cardioplegia. This improvement could not be ascribed to alterations in preload, afterload or body temperature. Similarly, Breisblatt et al (9) undertook serial hemodynamic and radionuclide ventriculography measurements in 24 patients undergoing uncomplicated elective coronary bypass surgery in whom intermittent blood cardioplegia was utilized. They demonstrated a significant depression in right and left ventricular ejection fraction and cardiac index which appeared maximal at four hours postoperatively but resolved within 24-48 hours. These and similar studies demonstrating postoperative stunning were carried out in low risk, elective patient populations where such transient contractile dysfunction can generally be readily reversed with appropriate inotropic support if necessary. Stunning may be significantly more problematic in high risk/complex situations or where there is concomitant myocardial necrosis.

The incidence of perioperative myocardial infarction reported in the literature is somewhat variable, at least in part because of the use of a variety of diagnostic methodologies and criteria. The combined incidence of Q and non-Q wave infarction in the surgical arm of the GUARDIAN trial ranged from 7.1 to 13.0% in the four study subgroups (10). Other clinical trials such as the Arterial Revascularization Therapies Study (n = 496) (11) and the Warm Heart Surgery Study (n = 1,732) (3) reported perioperative infarction rates of 11.5% and 12.3 - 17.3% respectively using enzymatic criteria. Where clinically appropriate criteria have been used to define perioperative infarction, studies have demonstrated significant detrimental effects on both early and longer term morbidity and mortality (12,13). Although perioperative infarction can result from a number of causes including acute vasospasm, coronary or graft thrombosis, technical error, or atheroembolism, suboptimal myocardial protection is likely of greatest significance.

3. CARDIOPROTECTIVE EFFECTS OF NHE-1 INHIBITION

As discussed in Chapters 15 and 18 of this volume, a large number of studies using a variety of models of myocardial ischemia/reperfusion injury have demonstrated a range of protective effects with NHE inhibition. These beneficial effects include infarct size reduction, a decrease in ventricular arrhythmias, a reduction in myocardial edema, enhanced ultrastructural and ATP preservation, a reduction in apoptosis, inhibition of the cardiotoxic effects of ischemic metabolites and improved recovery of both systolic and diastolic function. In addition to cardioprotective effects in acute ischemia, NHE inhibition has also more recently been shown to reduce the post-infarction remodeling process and to inhibit ventricular hypertrophy mediated by a variety of hypertrophic factors (Chapter 14).

The first study to demonstrate a cardioprotective effect with NHE inhibition was published by Karmazyn (14) using isolated rat hearts subjected to ischemia and reperfusion. Hearts treated with the nonspecific NHE inhibitor amiloride during both ischemia and reperfusion showed a significant improvement in recovery of systolic function as well as less release of creatine kinase during the reperfusion period. Numerous subsequent studies using both global and regional ischemia models have confirmed and expanded upon the findings of this initial study. We demonstrated (15) that rabbit hearts subjected to 1 hour of normothermic global ischemia showed significantly enhanced recovery of left ventricular force with almost complete prevention of reperfusion-induced contracture when treated with either amiloride or the amiloride analogue methylisobutyl amiloride prior to ischemia and throughout reperfusion. A subsequent study by Hendrikx et al (16) used HOE 694, a more selective NHE1 inhibitor with a structure distinct from amiloride, in blood-perfused rabbit hearts undergoing 45 minutes of global normothermic ischemia. This study also demonstrated improved recovery of systolic function and reduced contracture as well as better preservation of ATP and enhanced resynthesis of phosphocreatine. In addition, electron microscopy documented significantly better ultrastructural preservation in drug-treated hearts, including prevention of mitochondrial calcium aggregates.

Several studies have examined the use of NHE inhibitors in conjunction with cardioplegic solutions for myocardial protection. Using an isolated working rat heart model, Shipolini et al (17) demonstrated that the NHE-1 specific inhibitor cariporide produced improved recovery of cardiac output as compared to that seen with a standard cardioplegic solution alone. This enhanced recovery was significant both when drug was administered at the time of reperfusion or as an additive to the cardioplegic solution, although greater efficacy was seen with the latter approach. Improvement in functional recovery was inversely paralleled by decreases in post-reperfusion enzyme release, and was seen with normothermia as well as with moderate (28 °C) and profound (7.5°C) hypothermia. Another study using a surgical model of cardioplegic arrest (18) also showed significant improvement in functional recovery, a reduction in myocardial edema and significantly lower enzyme release in rat hearts receiving cariporide throughout perfusion or in the cardioplegic solution. Addition of drug to the reperfusate alone did not confer significant benefit apart from a modest decrease in enzyme release.

A recent study by Cox et al (19) used the selective NHE inhibitor EMD 96785 in dogs undergoing 2 hours of cold (4°C) crystalloid cardioplegic arrest while on cardiopulmonary bypass. Cardiac function as measured by preload recruitable stroke work following separation from bypass did not differ from the baseline pre-ischemic level in the drug-treated group, and was significantly better than that seen in the control group when measured at

30 and 60 minutes post-bypass. Both drug-treated and control groups showed increases in myocardial water content measured during and after cardiopulmonary bypass, but this increase was significantly less at each timepoint in the NHE inhibitor treated group. As pointed out in their discussion, there are several factors that contribute to interstitial edema in hearts undergoing cardioplegic arrest including a reduction in interstitial pressure with diastolic arrest, a cessation of myocardial lymph flow in the absence of contraction and a decrease in plasma oncotic pressure secondary to both crystalloid cardioplegia administration and bypass-associated hemodilution. As NHE inhibition would not be expected to affect these factors, it supports the concept that inhibition of the exchanger reduces intracellular water content by inhibiting intracellular sodium accumulation.

A further consideration with respect to the utilization of NHE inhibitors in cardiac surgery is the potential interaction with anaesthetic agents. Studies using an ischemia/reperfusion isolated rat heart model have suggested an additive protective effect resulting in superior recovery of function when cariporide is added to a number of anesthetic agents. (20).

4. MYOCARDIAL PROTECTION FOR TRANSPLANTATION

Optimal myocardial protection continues to be of paramount importance in cardiac transplantation. The safe limit for donor-heart ischemic time is generally considered to be in the range of 4-6 hours.

Data from the Registry of the International Society for Heart and Lung Transplantation continues to show that donor-heart ischemic time is a significant risk factor for both initial and long term recipient survival (21). Improvements in donor-heart preservation techniques that would extend the ischemic interval would have clear potential benefit with respect to immunologic, logistical, economic and clinical factors. For example, extension of safe donor heart ischemic time would facilitate organ sharing and transport, and would permit prospective HLA typing with potentially fewer rejection episodes. Improvements in myocardial preservation would facilitate hemodynamic recovery with potentially fewer perioperative complications and intensive care unit costs. In considering myocardial preservation for transplantation, there are a number of differences in comparison to most other cardiac surgical procedures. These include the use of profound hypothermia, significantly longer ischemic times, the use of a single cardioplegic solution infusion and the absence of any collateral perfusion. Although there has been interest in the use of continuous, low-flow hypothermic perfusion, most centres continue to use simple hypothermic storage following a single infusion of cardioplegic solution.

A number of studies have examined NHE inhibition as a protective strategy for hearts subjective to prolonged hypothermic storage. Kupriyanov et al (22) used amiloride in pig hearts subjected to 15 hours of 10°C ischemic storage and concluded that NHE activation appeared to contribute to ischemia/reperfusion damage. Functional recovery was generally improved in the amiloride group as was oxygen consumption and contractile reserve. Although there was no difference in post re-perfusion ATP levels, ^{31}P nuclear magnetic residence spectroscopy data showed improved recovery of phosphocreatine content in amiloride-treated hearts. Myers and Karmazyn (23) showed significantly enhanced recovery of systolic function in rabbit hearts subjected to 12 hours of 4°C storage treated with HOE 694. In addition, a marked decrease in post reperfusion contracture was seen regardless of whether drug was administered prior to ischemia or only at the time of reperfusion (Fig. 3). Kevelaitis et al (24) used an isolated rat heart model of 4 hours of hypothermic (4°C) storage to evaluate ischemic preconditioning, pharmacologic opening of the mitochondrial potassium-ATP channel and NHE inhibition as protective strategies for cardiac donor preservation. Their results support the concept that ischemic preconditioning and NHE inhibition result in myocardial protection through different mechanisms and that their beneficial effects are additive. Neither approach was beneficial with respect to endothelial dependent or independent vasomotor function.

The clinical relevance of these isolated heart protocols is reinforced by additional studies demonstrating significant cardioprotective effects in large animal models of orthotopic transplantation. Kim et al (25) examined the effect of both donor and recipient treatment with HOE 642 using a canine orthotopic transplantation model. Several protocols for drug administration were examined using hearts subjected to 24 hours of hypothermic storage in a hyperkalemic crystalloid cardioplegic solution. Treatment of both donor and recipient, as opposed to administration in the cardioplegic solution or to the recipient alone, resulted in improved myocardial compliance, lower myocardial water content and improved ultrastructure preservation. Most importantly from a clinical standpoint, all animals in the two groups in which both donor and recipient were treated with the NHE inhibitor were successfully weaned from cardiopulmonary bypass and all maintained a cardiac index compatible with survival. This was in marked contrast to the functional results in the other groups. Martin et al (26) supplied further evidence for the potential utility of NHE inhibition in cardiac transplantation using orthotopic transplantation in pigs after donor hearts underwent 30 minutes of normothermic ischemia. Despite this severe ischemic result, significantly improved recovery of ventricular function with supporting evidence of minimal ischemic damage on histologic examination was seen in the drug-treated group.

5. POTENTIAL FOR NHE-1 ACTIVATION DURING CARDIAC SURGERY

Although intracellular acidosis is the major stimulus to NHE activation, the activity of the exchanger can be increased through a variety of receptor and non-receptor mediated pathways. A number of factors that act through G-protein coupled receptors such as adrenergic stimulation, endothelin, angiotensin II and thrombin are of potential clinical significance in cardiac surgery.

Myocardial ischemia is known to result in sympathetic nervous system activation, as well as local tissue release of norepinephrine and enhanced $alpha_1$ adrenergic signaling. $Alpha_1$ adrenergic agents have been shown to increase NHE activity, specifically the $alpha_{1A}$ receptor sub-type (27). Conversely $beta_1$ adrenergic stimulation has been shown to inhibit NHE activity in some species, such that the net effect of catecholamines on NHE activity is undoubtedly complex. Nonetheless, NHE inhibition has been shown to counteract the exacerbation of post-ischemic contractile dysfunction (28) and reperfusion induced ventricular arrythmias (29) produced by $alpha_1$ adrenergic stimulation. This is of potential clinical relevance in that catecholamine levels are known to be significantly and persistently elevated in cardiac surgery patients. Furthermore agents that activate $alpha_1$ adrenergic receptors are not infrequently used clinically for inotropic or vasopresser support.

Systemic endothelin levels have also been shown to be signific-antly increased in patients undergoing CPB procedures and this constitutes another pathway whereby sarcolemmal NHE activity may be increased. The underlying mechanisms for this increase in endothelin include myocardial ischemia/reperfusion, reduced clearance during CPB secondary to a reduction in pulmonary blood flow, endothelial dysfunction secondary to altered perfusion, and increased synthesis due to thromboxane release from activated platelets (30). Clinical studies have suggested a detrimental effect in that higher levels of endothelin production during CPB have been associated with a greater need for postoperative inotropic support and a longer requirement for intensive care (31). Recent studies by Verma et al (32,33) provide important evidence linking endothelin to myocardial cell injury and micovascular constriction following ischemia/reperfusion, as well as data demonstrating exacerbation of these deleterious effects in the setting of hyperglycemia or diabetes. Khandoudi et al (34) have demonstrated in

rat hearts that endothelin-induced depression of post-reperfusion ventricular function can be prevented with NHE inhibition.

There is also evidence that NHE inhibition may provide protection from the cardiotoxic effects of a number of agents such as lysophosphatidylcholine (35) and hydrogen peroxide (36). Hydrogen peroxide may be of particular significance with respect to cardiac surgery. Myocardial levels of catalase and glutathione peroxidase, the enzymes that degrade hydrogen peroxide, are known to be decreased by ischemia (37) and plasma levels of hydrogen peroxide have been shown to increase significantly in cardiac surgery patients (38). Ferreira et al (39) reported that hydroperoxide-initiated chemiluminescence in myocardial biopsies obtained from patients undergoing revascularization procedures was significantly increased 10 minutes after reperfusion. Hydrogen peroxide has been shown to increase NHE activity via a mitogen-activated protein kinase pathway (40) while Myers et al (36) have demonstrated that the impaired recovery of function induced by hydrogen peroxide in a rat ischemia-reperfusion model can be largely prevented through concomitant treatment with an NHE inhibitor.

Much of the increase in circulating hydrogen peroxide appears to originate from neutrophils, primarily secondary to CPB-induced activation. It is of interest that the NHE-1 isoform is the predominant isoform in neutrophils as well as cardiomyocytes. Faes et al (41) demonstrated a protective effect from NHE inhibition in a rat heart model of neutrophil-induced reperfusion injury. In vitro neutrophil activation as assessed by chemiluminescence intensity was inhibited by MIA in a dose-dependent manner, and MIA pretreatment significantly enhanced postischemic recovery of left ventricular developed pressure in neutrophil-perfused hearts. Gumina et al (42) demonstrated that the decrease in infarct size seen with the selective NHE-I inhibitor BIIB-513 in a canine model of regional ischemia was accompanied by a reduction in the accumulation of neutrophils in the ischemic zone as determined by both histologic examination and myeloperoxidase activity levels. A significant decrease in phorbol-induced neutrophil activation was also seen, although there was no change in CD18 upregulation. A subsequent study (43) has demonstrated that the expression of intercellular adhesion molecules in coronary microvascular endothelial cells is significantly attenuated by NHE inhibition, thus demonstrating an additional mechanism whereby pharmacologic intervention to inhibit the exchanger may act to decrease ischemia/reperfusion injury.

6. CLINICAL STUDIES OF NHE-1 INHIBITION IN CARDIAC SURGERY

As discussed in Chapter 17, a number of clinical trials have been or are currently being carried out to assess efficacy of NHE-1 inhibitors in cardiac surgery. These include the completed GUARDIAN (**GUAR**d **D**uring Ischemia **A**gainst **N**ecrosis) trial (10) and the EXPEDITION (Na^+/H^+ **EX**change Inhibition to **P**revent **C**oronary **E**vents in Acute Cardiac Con**DITION**s) trial whose results will be released in Spring, 2003.

7. CONCLUSION

A wide range of experimental studies have contributed to the development of a variety of myocardial protective strategies used clinically in cardiac surgery. Although currently used techniques generally provide excellent results in elective low-risk procedures, optimal myocardial protection remains an important issue, particularly in higher risk clinical scenarios. The consistent efficacy of NHE inhibition in surgically relevant experimental models strongly suggests an important potential for clinical application in cardiac surgery.

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Chapter 20

NHE-1 INHIBITION: A POTENTIAL NEW TREATMENT FOR RESUSCITATION FROM CARDIAC ARREST

Raúl J. Gazmuri and Iyad M. Ayoub

Finch University of Health Sciences/The Chicago Medical School and the North Chicago VA Medical Center, Chicago, Illinois, USA

1. INTRODUCTION

Research on methods to improve the effectiveness of closed-chest resuscitation has traditionally centered on efforts to develop more effective means to deliver oxygen and energy substrates (1,2) and on efforts to limit injury associated with the resuscitation process (3,4). Yet, the possibility that cardiac resuscitation and survival may be improved by targeting specific pathogenic pathways that are activated during ischemia and reperfusion has only recently been considered. Among the various pathways that can play a pathogenic role during ischemia and reperfusion, activation of the sarcolemmal sodium-hydrogen exchanger isoform-1 (NHE-1) with subsequent Na^+-induced cytosolic Ca^{2+} overload has emerged as an important target (5-9).

Our ongoing research using animal models of ventricular fibrillation (VF) indicates that NHE-1 inhibition can markedly ameliorate myocardial abnormalities that limit resuscitability and, in turn, enhance the efficacy and outcome of closed-chest resuscitation (10-12).

In this chapter we first address the potential pathogenic role of NHE-1 during VF-induced cardiac arrest. We then discuss specific cardiac abnormalities that develop during resuscitation from VF and the effects of NHE-1 inhibition on these abnormalities. Finally we examine the

implications of these findings for resuscitation of humans after an episode of sudden cardiac death.

2. POTENTIAL ROLE OF NHE-1 DURING CARDIAC ARREST

Increased sarcolemmal Na^+ influx with consequent intracellular Na^+ accumulation due to inability of the Na^+-K^+ pump to extrude Na^+ has been recognized as an important pathogenic mechanism of myocardial cell injury during ischemia and reperfusion (Figure 1) (13,14). Accumulation of Na^+ becomes a "substrate" for reperfusion injury (15) and intensifies processes detrimental to cell function (see below). Na^+ can enter the cell during ischemia through the NHE-1, the Na^+-HCO_3^- cotransporter, and Na^+ channels; however, NHE-1 seems to be the predominant route of Na^+ entry under these abnormal conditions (16).

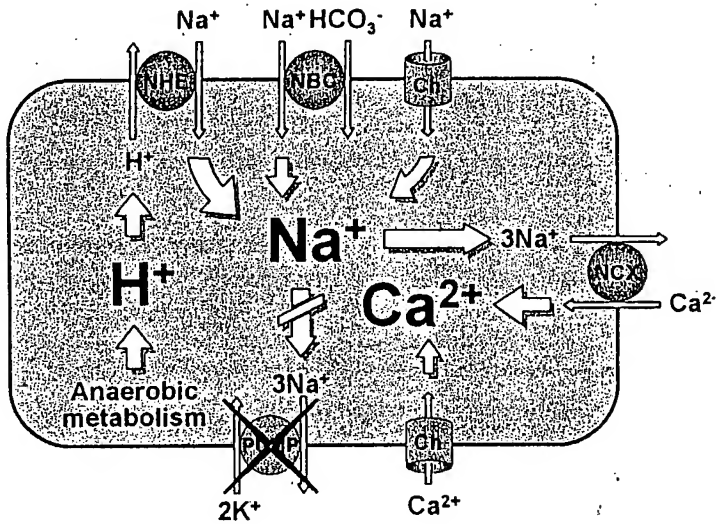

Figure 1: Anaerobic metabolism prompts intracellular acidosis, which activates the sarcolemmal sodium-hydrogen exchanger isoform-1 (NHE-1) promoting sarcolemmal Na^+ entry in exchange for H^+. Na^+ may also enter the cell through the Na^+-HCO_3^- cotransporter (NBC) and voltage-gated Na^+ channels (Ch). Because the activity of the Na^+-K^+ pump (PUMP) is disabled during ischemia, Na^+ accumulates, limiting Ca^{2+} extrusion and favoring Ca^{2+} entry through the Na^+-Ca^{2+} exchanger (NCX) acting in reverse mode. Ca^{2+} may also enter the cell through voltage-gated channels.

NHE-1 is activated by the profound intracellular acidosis that develops during ischemia (17), initiating an electroneutral sarcolemmal Na^+-H^+ exchange. It is thought that as protons exit the cell and accumulate in the extracellular space, the transsarcolemmal proton gradient declines, hence diminishing – but not eliminating – the Na^+-H^+ exchange (14).

Reperfusion with normo-acidic fluid washes out the acidic extracellular space and reestablishes a proton gradient that can intensify the sarcolemmal Na^+-H^+ exchange. When reperfusion occurs with normal flows, this pathogenic mechanism resolves promptly as aerobic metabolism and the activity of the Na^+-K^+ pump are restored (18), and the emergent pathways responsible for anaerobic acid production recede (19). However, during cardiac arrest when conventional closed-chest techniques are used, the coronary blood flow generated rarely exceeds 20% of normal (20). This flow is not sufficient to reverse ischemia (21) but sufficient to supply the coronary circuit with normo-acidic blood (22). Thus, the presence of unremitting ischemia and a large transsarcolemmal proton gradient create conditions favorable for NHE-1 to remain active during the resuscitation effort and probably the initial minutes following the return of spontaneous circulation. During this early post-resuscitation period, additional injury may develop as intracellular acidosis is reversed, unopposing adverse effects of Na^+-induced cytosolic Ca^{2+} excess on structural and functional proteins.

In addition to these local mechanisms, cardiac arrest triggers a prominent neuroendocrine stress response, releasing mediators that can further enhance NHE-1 activity. For example, activation of α_1-adrenergic (23), endothelin-1 (24), and angiotensin II (25) receptors has been shown to increase the proton sensitivity of the exchanger by activation of phospholipase C and subsequent phosphorylation of NHE-1, hence increasing the Na^+-H^+ exchange activity for a given intracellular pH level (26). In addition, metabolites produced during ischemia and reperfusion such as hydrogen peroxide (27) and lysophosphatidylcholine (28) can also activate NHE-1.

The mechanism by which Na^+ accumulation worsens ischemia and reperfusion injury is the subject of current research. Several observations suggest that cytosolic Na^+ overload could have potential detrimental effects on energy metabolism by intensifying the Na^+-K^+ pump activity during attempts to extrude the cytosolic Na^+ excess (29). In addition, compelling evidence points to Na^+-induced sarcolemmal Ca^{2+} entry through the Na^+-Ca^{2+} exchanger (NCX) acting in its reverse mode as a major mechanism of injury (15,30-33). Cytosolic Ca^{2+} overload is known to disrupt cell function by adversely affecting structural and functional proteins. Moreover, recent studies suggest that Ca^{2+} overload in conjunction with ATP depletion and oxidative stress can disrupt mitochondrial function causing mitochondrial permeability transition (34,35). This phenomenon is attributed to opening of

a non-specific high-conductance pore in the inner mitochondrial membrane and is characterized by mitochondrial swelling, depolarization, and uncoupling (36).

3. MYOCARDIAL EFFECTS OF VENTRICULAR FIBRILLATION

During VF the myocardial energy requirements are comparable to or even greater than those of the normally beating heart (37,38). Consequently, when VF precipitates cardiac arrest and coronary blood flow ceases, a severe energy imbalance develops, leading – within minutes – to intense myocardial ischemia and profound myocardial acidosis (39).

In an isolated rat heart preparation, in which global myocardial ischemia was produced by reducing the coronary perfusion flow, VF precipitated ischemic contracture and caused post-ischemic diastolic and systolic dysfunction (38). In the same studies, VF increased coronary vascular resistance to approximately 200% of baseline, presumably as a result of decreased luminal distending forces (less coronary flow) and external compression by the fibrillatory activity (40). However, under identical low flow conditions, but in the absence of VF, ischemic contracture did not occur, the coronary vascular resistance increased to 150% of baseline, and only transient and minimal post-resuscitation myocardial dysfunction ensued (38).

In addition to these metabolic and mechanical effects, activation of voltage-gated channels during VF may promote additional sarcolemmal Na^+ and Ca^{2+} entry and accentuate ischemic injury (41,42). In our isolated rat heart preparation we examined changes in intramyocardial Na^+ during ischemia and VF using atomic absorption spectrophotometry (10). Control hearts had an intramyocardial Na^+ content of 11.5 ± 0.8 mmol/kg wet tissue. However, after 25 minutes of ischemia (including 15 minutes of low coronary perfusion) the Na^+ content had increased to 15.1 ± 1.7 mmol/kg in the absence of VF and to 20.2 ± 2.1 mmol/kg in the presence of VF. In subsequent pilot studies conducted in collaboration with Dr. Rolf Brandes at Loyola University (unpublished), the effects of VF on cytosolic Ca^{2+} were examined using surface spectrofluorometry in isolated rat hearts loaded with indo-1. In these pilot studies, induction of VF during baseline perfusion increased diastolic Ca^{2+} without major effects on systolic Ca^{2+}. Yet, after interruption of coronary perfusion, the mean Ca^{2+} rose to levels exceeding baseline systolic Ca^{2+} levels (Figure 2).

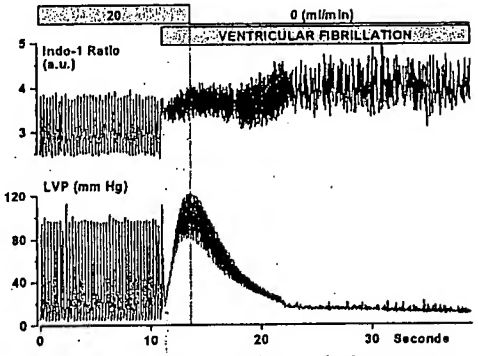

Figure 2: LVP = Left ventricular pressure. Effects of VF on cytosolic Ca^{2+} expressed in arbitrary units (a.u.) during normal perfusion and during zero coronary flow. Measurements were obtained in a crystalloid perfused isolated rat heart preparation loaded with indo-1.

These data suggest that during the global myocardial ischemia of cardiac arrest, VF may worsen ischemic injury by intensifying energy deficit and by accentuating intracellular Na^+ and Ca^{2+} overload. A pathogenic model relating VF, ischemia, and NHE-1 is depicted in Figure 3.

4. NHE-1 INHIBITORS

The initial evidence suggesting that sarcolemmal Na^+-H^+ exchange could play an important pathogenic role during myocardial ischemia and reperfusion was reported by Dr. Morris Karmazyn using non-specific NHE inhibitors such as amiloride and 5-amino substituted derivatives (5,43). Within the past decade, more selective inhibitors – structurally less related to amiloride – have been developed. These new compounds are also more potent and with no apparent effects on other ion transport or pH regulatory systems. There is extensive literature on the benzoylguanidine derivatives HOE-694 and HOE-642 (cariporide), demonstrating consistent myocardial protection during ischemia and reperfusion (44,45). Cariporide has been shown to be highly selective for the isoform 1 without apparent effects on the Na^+-Ca^{2+} exchanger or the fast Na^+ current. Cariporide has only negligible biological actions on non-inactivating Na^+ currents but these effects are observed only at very high concentrations (44).

Within the past few years, new NHE-1 inhibitors such as TY-12533 (46), KB-R9032 (47), SL 59.1227 (48), SM-20550 (49), eniporide (50), and

zoniporide (51) have been developed. These compounds are structurally diverse but exert similar myocardial protective actions.

NHE-1 inhibitors have been investigated in patients with acute coronary syndromes undergoing emergent coronary interventions (52,53). In the study by Rupprecht and coworkers, global and regional left ventricular function were improved in patients presenting with acute myocardial infarction who received 40 mg of cariporide before percutaneous coronary angioplasty (52). In the GUARDIAN trial, cariporide had no significant effects on overall mortality; however, it improved outcome in a smaller subset of patients undergoing coronary artery bypass graft who received a higher dose of cariporide (53). The results of this trial prompted the ongoing multicenter EXPEDITION trial. Studies on the effects of NHE-1 inhibition during resuscitation from cardiac arrest in humans are eagerly awaited.

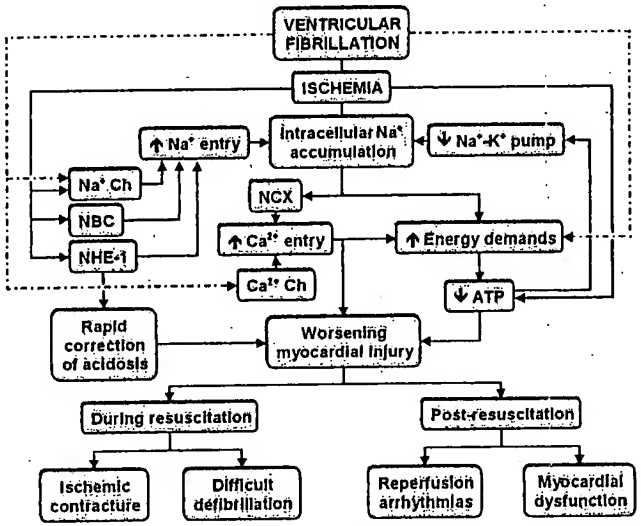

Figure 3: During ischemia, Na^+ may enter the cell through the NHE-1 (activated by intracellular acidosis), the Na^+-HCO_3^- co-transporter (NBC), and Na^+ channels. Na^+ extrusion by the Na^+-K^+-pump is progressively curtailed as the ATP deficit intensifies during ischemia. VF favors additional Na^+ entry through voltage-gated Na^+ channels and accentuates the energy deficit (fibrillatory activity) further limiting Na^+ extrusion. The intracellular Na^+ excess prompts Ca^{2+} entry through the Na^+-Ca^{2+} exchanger (NCX) acting in reverse mode. In addition, Ca^{2+} may also enter the cell through voltage-gated channels. The combination of Na^+ and Ca^{2+} overload along with ATP depletion (and rapid normalization of intracellular acidosis during reperfusion) worsens myocardial ischemic injury contributing to the development of abnormalities that can limit resuscitability.

5. MYOCARDIAL ABNORMALITIES DURING VF AND EFFECTS OF NHE-1 INHIBITION

The intense myocardial ischemia that develops during VF and the subsequent injury associated with reperfusion are associated with the development of myocardial abnormalities that can limit the effectiveness and outcome of closed-chest resuscitation. These myocardial abnormalities are herein discussed along with the experimental evidence supporting a favorable effect of NHE-1 inhibition.

5.1 Ischemic Contracture And Closed-Chest Resuscitation

Ischemic contracture refers to progressive increases in myocardial wall thickness with reductions in ventricular cavity that develops as a result of severe myocardial ischemia. Experimentally, onset of ischemic contracture is preceded by cytosolic Ca^{2+} increases and coincides with reductions in ATP levels to less than 10% of normal (54). Ischemic contracture can progress to an irreversible state described by Dr. Denton Cooley and colleagues as "stone heart" more than 30 years ago (55). More recently, ischemic contracture has been shown to develop during cardiac arrest in animal models of VF (56,57) and in human victims during open-chest resuscitation after failure of closed-chest resuscitation (58). Because ischemic contracture progressively reduces the left ventricular cavity, it limits the amount of blood available for ejection during cardiac compression. This effect partly explains time-dependent reductions in coronary perfusion pressure that in turn can compromise resuscitability.

Our studies suggest that within the relatively short time interval of a typical episode of cardiac arrest and resuscitation, ischemia alone is not sufficient to precipitate ischemic contracture. Under these conditions, both VF and myocardial reperfusion are required (38). This last requirement suggests that reperfusion has a permissive effect on the genesis of ischemic contracture.

Our research further indicates that NHE-1 inhibition can markedly attenuate ischemic contracture (10,11). In an isolated isovolumic rat heart model of VF, NHE-1 inhibition (using cariporide) attenuated increases in left ventricular pressure during the interval of VF and low-flow myocardial ischemia (Figure 4) (11). In an intact rat model of VF and closed-chest resuscitation, this effect was associated with less depth of chest compression required to attain the same coronary perfusion pressure as in control rats (11). In more recent studies using a pig model of VF and closed-chest

resuscitation instrumented with a transesophageal echo-Doppler probe, cariporide prevented increases in left ventricular wall thickness during VF and enabled chest compression to generate and maintain a coronary perfusion pressure above resuscitability thresholds throughout the resuscitation effort (59).

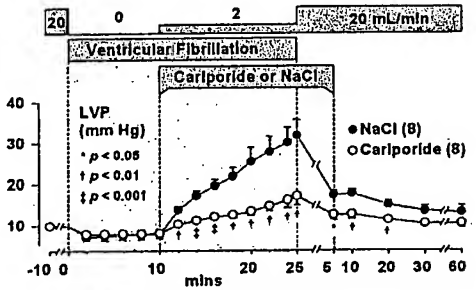

Figure 4: Left ventricular pressure (LVP) during VF in isolated perfused rat hearts. During normal perfusion, LVP was measured at the end of diastole. Adapted with permission from Gazmuri and collaborators (*Circulation* 2001;104:234-9).

5.2 Myocardial Perfusion And Coronary Vascular Resistance

Reestablishment of coronary blood flow above critical thresholds is paramount for successful cardiac resuscitation (20,60,61). Because the intense myocardial ischemia that develops during cardiac arrest promotes maximal coronary vasodilation, coronary blood flow during closed-chest resuscitation becomes a linear function of the pressure gradient between the aorta and the right atrium at the end of chest relaxation; i.e., coronary perfusion pressure (62,63). During chest compression, comparable increases in aortic and right atrial pressures essentially preclude flow across the coronary circuit (64). Experimentally, increases in coronary perfusion pressure above 10 or 20 mm Hg, contingent on the animal species, correlate with restoration of spontaneous circulation in more than 80% of the instances (60,65,66). In humans, a minimum threshold level of 15 mm Hg has been reported (61).

Notwithstanding maximal coronary vasodilation, increases in coronary vascular resistance occur during ischemia and VF (38,40). As already pointed out, this effect probably results from lower luminal distending pressures and increased myocardial fibrillatory activity, thus reflecting

decreases in coronary transmural pressure independently of any intrinsic effect on coronary smooth muscle tone. The possibility that NHE-1 inhibition could ameliorate such increases in coronary resistance and cause a leftward shift of the pressure-flow relationship has been suggested by observations made in our isolated rat heart model of VF (11). In these studies, increases in coronary vascular resistance during ischemia and VF were significantly ameliorated by cariporide (Figure 5). Applying these observations to our *in vivo* model (in which chest compression was titrated to maintain a target coronary perfusion pressure of approximately 25 mm Hg), an impressive 36% increase in coronary blood flow could result from NHE-1 inhibition. This effect, in and of itself, could contribute to mitigate the severity of ischemic injury. Experiments using fluorescent microspheres to assess effects on myocardial blood flow are underway in our rat model of closed-chest resuscitation.

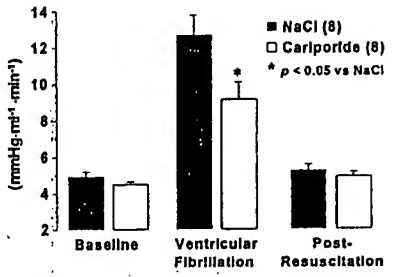

Figure 5: Coronary vascular resistance in a crystalloid-perfused isolated rat heart preparation at baseline (flow = 20 ml/min), during VF (flow = 2 ml/min), and after resuscitation (flow = 20 ml/min). Data obtained from studies shown in Figure 4.

5.3 Timing For Electrical Defibrillation

Delivery of electrical shocks immediately upon recognition of VF is considered pivotal for successful resuscitation from cardiac arrest (67). This approach is supported by several studies in which impressive resuscitation outcomes have been reported after implementation of programs for early defibrillation by first responders (68,69). However, studies in dogs by Niemann and coworkers (70) and in patients by Cobb and coworkers (71) have suggested that attempts to immediately defibrillate may become less successful as the duration of untreated VF increases. Under these

conditions, a period of chest compression appears to improve the responsiveness to electrical shocks.

In recent studies using a rat model of VF and closed-chest resuscitation, we reported that after a period of 10 minutes of untreated VF approximately 6 minutes of chest compression were required to maximize the likelihood that electrical shocks could successfully restore spontaneous circulation (72). In these studies, analysis of VF amplitude and frequency characteristics demonstrated that successful defibrillation was preceded by a gradual return of VF waveform characteristics to those present immediately after induction of VF.

These observations suggest that metabolic abnormalities that develop during the interval of untreated VF need to be reversed – or at least ameliorated – before electrical shocks can successfully terminate VF and restore spontaneous circulation. This effect is currently accomplished by promoting flow across the coronary circuit. Whether additional benefit can be obtained from concurrent metabolic interventions is unclear. Studies are awaited to determine whether NHE-1 inhibition during this "obligatory" period of chest compression could facilitate successful defibrillation.

5.4 Ventricular Arrhythmias After Return Of Spontaneous Circulation

Electrical instability with recurrent episodes of VF commonly occur after resuscitation from cardiac arrest (73) and may in part account for the nearly 30% incidence of early post-resuscitation deaths (74). The mechanisms involved are likely to be similar to those associated with reperfusion after coronary occlusion. In this setting, ventricular arrhythmias are accompanied by prominent repolarization abnormalities that include shortening of the action potential (AP) duration, change in AP morphology to a more triangular shape, decreased AP amplitude, AP duration alternans, and afterdepolarizations (75,76). These abnormalities are typically reversible and occur during the initial 5 to 10 minute after reperfusion, coincident with the period of maximal ventricular ectopic activity. Shortening of the AP duration is in part related to opening of sarcolemmal K^+_{ATP} channels (77). However, recent evidence suggests that AP shortening may be associated with NHE-1 activation during ischemia and reperfusion (78).

In our rat and pig models of VF, prominent ventricular ectopic activity typically occurs within the initial 5 to 10 minutes after return of spontaneous circulation and is accompanied by episodes of recurrent VF that require additional defibrillation attempts. Administration of cariporide had an impressive effect in both rats and pigs, markedly reducing post-resuscitation

ventricular ectopic activity and fully preventing recurrent episodes of VF (Figure 6). In recent studies in pigs, we have documented marked shortening of the AP duration during the post-resuscitation interval, which was almost completely prevented by administration of cariporide (59).

Thus, NHE-1 inhibition appears to be a highly effective intervention for ameliorating repolarization abnormalities and preventing life-threatening ventricular arrhythmias during the early post-resuscitation interval.

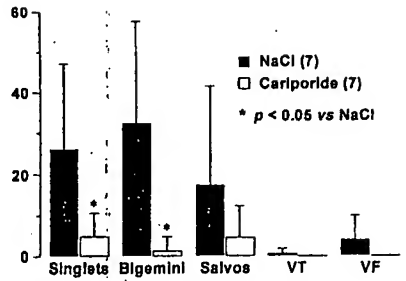

Figure 6: Ventricular ectopic activity during the initial 5 minutes post-resuscitation in a pig model of closed-chest resuscitation.

5.5 Post-Resuscitation Myocardial Dysfunction

Variable degrees of global diastolic and systolic dysfunction have been documented in animal models and in human victims after resuscitation from cardiac arrest (79-81). Myocardial dysfunction typically reverses within hours or days. However, if sufficiently severe it may preclude restoration of stable circulation and also contribute to early post-resuscitation deaths. Diastolic dysfunction is characterized by left ventricular wall thickening with reductions in end-diastolic ventricular cavity size that is more prominent early after return of spontaneous circulation (59,82), and probably reflects resolving ischemic contracture. Systolic dysfunction is characterized by decreases in contractility with reductions in ejection fraction, stroke volume, and cardiac output. The combination of diastolic and systolic dysfunction is particularly problematic because increased myocardial stiffness may preclude the compensatory ventricular dilatation required for overcoming decreases in contractility at the very vulnerable early post-resuscitation period. Studies in our rat and pig models of VF indicate that NHE-1 inhibition can prevent diastolic dysfunction and ameliorate systolic dysfunction, favoring earlier restoration of stable circulation (11).

6. CLINICAL IMPLICATIONS

It is estimated that in the United States alone, approximately 350,000 individuals suffer an episode of cardiac arrest every year. Yet, less than 5% survive and return to productive lives (83). Thus, interventions that can increase this dismal outcome – even by a small fraction – could have a dramatic public health effect, saving thousands of lives.

A large and growing body of knowledge already supports the rationale of NHE-1 inhibition for ameliorating myocardial ischemic and reperfusion injury. The application of this knowledge to the cardiac arrest setting supports a potentially important role for NHE-1 inhibition, in which the effectiveness and outcome of closed-chest resuscitation could be improved by ameliorating ischemic contracture, reperfusion arrhythmias, and post-resuscitation myocardial dysfunction.

The beneficial effects of NHE-1 inhibition seem not to be limited by species differences (84). Sarcolemmal NHE-1 is expressed in human myocardium (85) and clinical trials have demonstrated capability of NHE-1 inhibition to ameliorate myocardial injury in selected groups of patients undergoing emergent coronary interventions (52,53). Thus, effects similar to those herein reported in rat and pig models could also apply to human victims of cardiac arrest and facilitate closed-chest resuscitation from VF. Clinical studies on NHE-1 inhibition during cardiac resuscitation are timely and eagerly awaited.

ACKNOWLEDGEMENTS

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INDEX

C

D

E

F

G

H

I

K

L

M

N

O

P

R

S

T

U

V

X

W

Z